

Functional insights into the plant-specific SR45 splicing factor

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ABSTRACT

Alternative splicing is a post-transcriptional regulatory mechanism that generates transcriptome and proteome diversity. The SR protein family of splicing regulators is essential for the execution and regulation of both constitutive and alternative pre-mRNA splicing. The SR-related protein SR45 is an *Arabidopsis* bona fide splicing factor that plays a key role in plant development as well as in sugar and abscisic acid (ABA) signaling during early seedling development. The *Arabidopsis* 5PTase13, belonging to a group of inositol phosphatases involved in plant growth, development and stress responses, is a splicing target of SR45 previously implicated in hormonal and light signaling pathways. The *5PTase13* gene is alternatively spliced, generating two isoforms shown here to be ubiquitously expressed in *Arabidopsis*. The 5PTase13 loss-of-function mutant we isolated exhibits shorter roots when grown under low light conditions. Moreover, overexpression of either the *5PTase13.1* or the *5PTase13.2* isoforms in the *sr45-1* background enhances the glucose hypersensitivity during early seedling development conferred by the *sr45-1* mutation. Finally, confocal microscopy data suggests that both 5PTase13 isoforms are located in the cytoplasm, with 5PTase13.1 appearing mostly associated with the plasma membrane. This work provides new insight into the *in vivo* roles of the SR45 splicing factor and its functional connections to the 5PTase13 phosphatase.

Keywords: 5PTase13, ABA, alternative splicing, *Arabidopsis*, glucose, SR45

RESUMO

O *splicing* alternativo é um mecanismo de regulação pós-transcricional que gera diversidade transcritômica e proteômica. As proteínas SR desempenham um papel essencial na execução e regulação do *splicing* constitutivo e alternativo do pré-mARN. A proteína SR45 é um factor de *splicing* genuíno em *Arabidopsis*, desempenhando um papel chave no desenvolvimento da planta e na sinalização dos açúcares e do ácido abscísico (ABA) durante o desenvolvimento inicial da plântula. A fosfatase 5PTase13 de *Arabidopsis*, pertencente a um grupo de inositol fosfatases envolvidas no crescimento e desenvolvimento vegetal bem como respostas ao stress, é um alvo de *splicing* da SR45 tendo sido previamente implicada nas vias de sinalização hormonais e da luz.

O gene *5PTase13* sofre *splicing* alternativo, gerando duas isoformas aqui descritas como sendo ubiquamente expressas em *Arabidopsis*. O mutante *knockout* de *5PTase13* que isolámos apresenta raízes mais curtas em condições de baixa luminosidade. Além disso, a sobre-expressão das isoformas *5PTase13.1* ou *5PTase13.2* no *background* do mutante *sr45-1* aumenta a hipersensibilidade à glucose durante o desenvolvimento inicial da plântula, fenótipo este conferido pela mutação *sr45-1*. Por fim, dados de microscopia confocal sugerem que ambas as isoformas da 5PTase13 se localizam no citoplasma com a 5PTase13.1 parecendo mais associada à membrana plasmática. Este trabalho fornece novas perspectivas sobre as funções *in vivo* do fator de *splicing* SR45 e suas conexões funcionais à fosfatase 5PTase13.

Palavras-chave: 5PTase13, ABA, *Arabidopsis*, glucose, *splicing* alternativo, SR45

RESUMO-ALARGADO

A maioria dos genes dos eucariotas autotróficos contém regiões codificantes, denominadas exões, interrompidas por regiões não-codificantes, os intrões. *Splicing* é o processo pelo qual os intrões do pre-mARN são removidos e os exões ligados entre si, de forma a obter um mARN maduro. Através do *splicing* alternativo múltiplas formas de mARN maduro podem ser obtidas a partir de um único pre-mARN. Assim, um único gene pode produzir mais que uma proteína originando diversidade transcritómica e proteómica. Em plantas, estima-se que 61% dos genes com intrões sofram *splicing* alternativo, tendo a sua relevância biológica vindo a revelar-se nos últimos anos, nomeadamente numa variedade de processos fisiológicos e de desenvolvimento.

As proteínas SR (ricas em serina e arginina) constituem uma das famílias mais conservadas de factores de *splicing*, sendo essenciais à execução e regulação do *splicing* constitutivo e alternativo do pre-mARN. Ao serem capazes de se ligar ao ARN e interagir com componentes do spliceossoma direccionando-os para os sítios de *splicing*, têm um papel crucial no *splicing* alternativo, determinando a escolha dos sítios de *splicing* em função da sua concentração. O factor de *splicing* específico de plantas SR45, recentemente excluído da família de proteínas SR após uma revisão da definição da nomenclatura desta família, desempenha um papel-chave no normal desenvolvimento da planta, bem como na sinalização de açúcares e da fitohormona ácido abscísico (ABA).

Em plantas, embora o conhecimento de um grupo específico de inositol fosfatases denominado 5PTases ainda seja limitado, estas estão implicadas em diversas funções relacionadas com o crescimento e desenvolvimento vegetal, bem como a resposta ao stress ambiental. Estudos de caracterização funcional da fosfatase 5PTase13 em *Arabidopsis* indicam a sua importância no desenvolvimento vegetal bem como em vias de sinalização hormonais e de luz, tendo também sido implicada na sinalização de nutrientes/energia. O gene que codifica esta fosfatase sofre *splicing* alternativo, originando duas isoformas distintas como resultado da retenção de um intrão na isoforma *5PTase13.1*. Neste trabalho, mostramos que ambas as isoformas são ubiquamente expressas em plântulas, tecidos aéreos e raízes de *Arabidopsis*. Verificamos também que a isoforma *5PTase13.2* é o transcrito mais abundante em plântulas e nos diferentes tecidos analisados.

Estudos anteriores mostraram que a *5PTase13* era significativamente induzida por ABA e ferimentos, e ligeiramente induzida pelo frio e sal, sugerindo o seu envolvimento na resposta ao stress. De facto, demonstrou-se também que mutantes *knockout* da *5PTase13* apresentam alterações na resposta ao stress induzido por ABA e a açúcares. Um dos objectivos do presente trabalho foi verificar se a produção das duas isoformas da *5PTase13* tem impacto funcional na resposta das plantas aos

açúcares e ABA. Começámos por isolar um novo mutante *knockout* da 5PTase13, *5ptase13-3*, e verificámos que em condições de pouca luz o mutante exhibe raízes mais curtas.

Este trabalho veio confirmar também que o gene *5PTase13* é um alvo do factor de *splicing* SR45 e quisemos investigar se as isoformas da 5PTase13 teriam algum efeito no modo de acção da SR45. Verificámos que plantas transgénicas sobre-expressando cada uma das isoformas da *5PTase13* no mutante *sr45-1*, exibem um fenótipo de hipersensibilidade à glucose mais acentuado do que o previamente descrito para o mutante *sr45-1* relativamente à germinação e desenvolvimento inicial de plântulas.

O último objectivo do presente trabalho foi estudar a localização subcelular de cada uma das isoformas da 5PTase13. Observámos através de microscopia confocal que ambas as isoformas se localizam no citoplasma e que a isoforma 5PTase13.1 poderá estar mais associada à membrana plasmática do que a 5PTase13.2.

Este trabalho fornece novas perspectivas relativamente às funções desempenhadas pelo factor de *splicing* SR45 e suas ligações funcionais à fosfatase 5PTase13.

ABBREVIATIONS LIST

ABA	abscisic acid
EEP	exonuclease/endonuclease/phosphatase
ESR	exonic splicing regulator
GFP	green fluorescent protein
HXK	hexokinase
Ins(1,4,5)P₃	inositol (1,4,5) triphosphate
Ins(1,3,4,5)P₄	inositol (1,3,4,5) tetrakisphosphate
ISR	intronic splicing regulator
mRNA	messenger RNA
NMD	nonsense-mediated decay
PLC	phospholipase C
pre-mRNA	precursor-mRNA
PTC	premature termination codon
PtdIns(3,4,5)P₃	phosphatidylinositide (3,4,5) triphosphate
PtdIns(4,5)P₂	phosphatidylinositide (4,5) biphosphate
PyTract	polypyrimidine tract
RNP	ribonucleoprotein
ROS	reactive oxygen species
RRM	RNA recognition motif
RS	arginine/serine-rich
RT-PCR	reverse-transcription polymerase chain reaction
S/RS	serine and arginine serine-rich
SNF1	sucrose non-fermenting 1
SnRK	SNF1-related kinase
snRNP	small nuclear ribonucleoprotein
SPXR	serine-proline-x-arginine motif
SF	splicing factor
SR	serine/arginine-rich
SS	splice site
TAIR	The Arabidopsis Information Resource
U2AF	U2 auxiliary factor
UTR	untranslated region

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I. INTRODUCTION

1. Pre-mRNA splicing in plants

Most genes in autotrophic eukaryotes contain coding regions, named exons, interrupted by stretches of non-coding sequences, called introns. Splicing is a fundamental post-transcriptional gene regulation process that removes the introns from the precursor-mRNA (pre-mRNA) and joins the exons in order to obtain a mature mRNA that may later be translated into protein. This process occurs cotranscriptionally and is carried out by a large ribonucleoprotein (RNP) complex, the spliceosome, composed of five small nuclear ribonucleoproteins (snRNPs) and a large number of non-snRNP proteins. There are four intron-defining splicing signals that are important for accurate splicing of the pre-mRNA – the 5' and 3' splice sites (SSs) with the consensus sequences GU and AG, respectively, that mark the border between exons and introns; the branch point, a sequence near the 3'SS that is defined by an adenoside residue; and the polypyrimidine tract (PyTract), a stretch of pyrimidines downstream of the branch point site (Figure 1). These consensus sequences are crucial in the splicing process, as a change in only one of the conserved nucleotides may inhibit intron splicing.

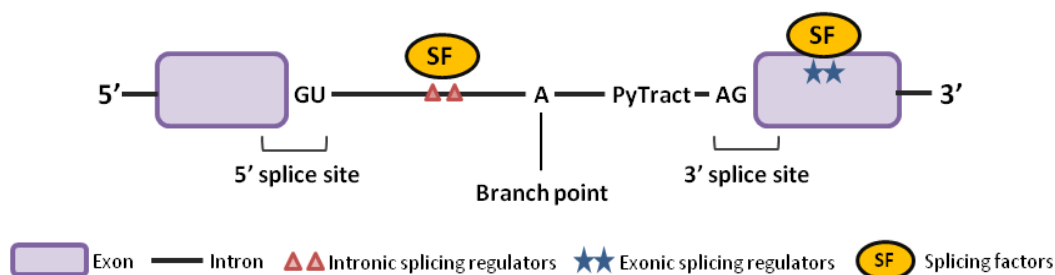


Figure 1. Regulatory elements that contribute to the regulation of alternative splicing

Pre-mRNA splicing is directed by *cis* elements, including the splice sites, the branch point and the polypyrimidine tract. The selection of alternative splice sites is affected by *trans*-acting factors binding to auxiliary exonic and intronic *cis* splicing regulators.

The splicing reaction involves two sequential *trans*-esterification reactions. First, there is the nucleophilic attack by the hydroxyl group of the adenosine in the branch point site to the 5' SS phosphate. This will create a bond between the first nucleotide of the intron and the branch point's adenosine, forming a looped structure known as lariat, and leaving a free 3' hydroxyl group at the 5' exon. The second reaction involves another nucleophilic attack of the newly freed 3' hydroxyl group to the phosphodiester at the 3' SS, which results in the junction of the two exons and the release of the lariat containing the intron.

Alternative splicing occurs when different combinations of splice sites are recognized by the spliceosome, thus allowing for different rearrangements of the genes' coding fragments and resulting in the generation of multiple forms of mature mRNA from the same pre-mRNA molecule. In this way, a single gene may lead to the production of more than one polypeptide, upscaling the genome's coding capacity. It is indeed the most prevalent mechanism to generate transcriptome and proteome diversity in metazoans (Graveley, 2001; Maniatis & Tasic, 2002). The most fascinating example is the *Dscam* gene from *Drosophila melanogaster* that can give rise to over 38000 alternatively-spliced variants (Schmucker et al., 2000). Alternative splicing may therefore have important consequences to the cell. It allows a single gene to give rise to multiple proteins, with different sequence and domain arrangement and hence potentially different subcellular localization, stability or function (reviewed in Syed et al., 2012). Moreover, it can also regulate transcript levels by introducing premature termination codons (PTCs) that then target the mRNAs for degradation by nonsense-mediated decay (NMD) (reviewed in Nicholson et al. 2010), a surveillance mechanism that prevents accumulation of truncated and potentially harmful proteins. It has been shown that alternative splicing coupled to NMD potentially regulates 13% of intron-containing Arabidopsis genes (Kalyna et al., 2012).

Alternative splicing can be constitutive or subject to regulation. In the first case, multiple isoforms are produced in constant amounts in all or in the majority of cell types, while in the latter case, different splicing patterns occur according to different cell environmental conditions. In fact, regulated alternative splicing can be tissue-specific, dictated by different developmental stages or modulated by external stimuli, with splice site usage being controlled by fundamental biochemical mechanisms, such as spliceosome composition and concentration of splicing factors, as well as chromatin structure and transcription rates (reviewed in Nilsen and Graveley, 2010; Staiger & Brown, 2013).

Most alternative splicing events can be classified into four basic types – exon skipping/inclusion, selection of alternative 5' or 3' splice sites and intron retention (Figure 2). In Arabidopsis the most prominent alternative splicing event is intron retention (~ 40%), followed by alternative 3' splice site and 5' splice site, with exon skipping events being relatively rare (Figure 2) (reviewed in Carvalho et al. 2013; Reddy et al., 2013). By contrast, in humans intron retention is the least frequent (<5%) event and exon skipping the most common (>40%) (Figure 2) (reviewed in Carvalho et al. 2013; Reddy et al., 2013).

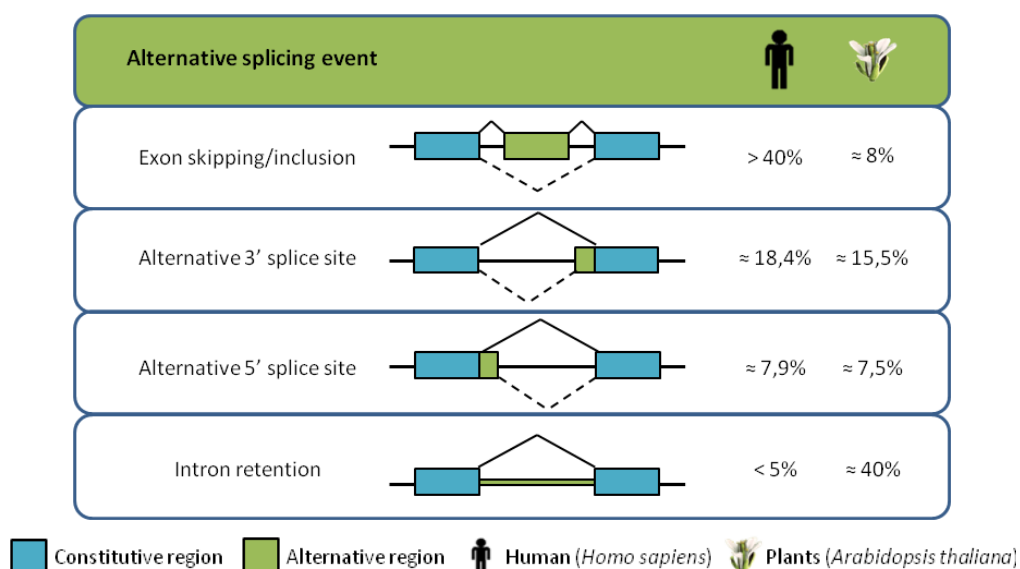


Figure 2. Frequency of the basic types of alternative splicing events in humans and Arabidopsis

Comparison of the proportion of the basic types of alternative splicing events between humans and Arabidopsis. Adapted from Reddy et al. (2013).

In the last decade, there have been great improvements regarding alternative splicing analyses, particularly in plants, where studies had been limited to individual genes. Up to 95% of human multiexon genes are estimated to undergo alternative splicing (Pan et al., 2008). In plants, recent high coverage RNA-seq transcriptome analyses estimated that at least 61% of intron-containing genes of Arabidopsis (Marquez et al., 2012), 48% of rice, *Oryza sativa* (Lu et al., 2010) and 30% of grape, *Vitis vinifera* (Vitulo et al., 2014) undergo alternative splicing. With the sequencing of other plant genomes, and using expressed transcript sequence alignments, it is also estimated that alternative splicing occurs in 55% of barley, *Hordeum vulgare* (International Barley Genome Sequencing et al., 2012), 40% of maize, *Zea mays* (Thatcher et al., 2014), 5% of potato, *Solanum tuberosum* (Potato Genome Sequencing et al., 2011) and 6% of the grass *Brachypodium distachyon* (Walters et al., 2013) genes. These estimates are bound to rise as RNA-seq data becomes available from various cell types, organs, and developmental stages, and from plants grown under different biotic and abiotic stresses. In humans, the misregulation of alternative splicing can lead to developmental abnormalities and a large number of diseases, such as breast and ovarian cancer, spinal muscular atrophy (SMA), cystic fibrosis or Alzheimer's disease (reviewed in Blencowe, 2006; Tazi et al., 2009; Venables et al., 2009; Ward & Cooper, 2010). Therapeutic strategies based on modulating alternative splicing are currently being developed to lighten the symptoms of some of these diseases (Nlend Nlend et al., 2010). In plant systems the biological relevance of alternative splicing is just beginning to unfold, with the last couple of years having seen an increasing number of examples of functional alternative splicing events in plants. Such reports have implicated alternative

splicing in a variety of developmental and physiological processes, such as photosynthetic and starch metabolism, hormone signaling, seed germination, root growth, flowering, circadian clock, natural variation, as well as in biotic and abiotic stress responses (reviewed in Carvalho et al., 2013; Staiger & Brown, 2013; Reddy et al., 2013).

The consensus sequences at the splice and branch point sites essential for splice site recognition are short and degenerate. Therefore, other *cis*-acting regulatory sequences in exons (exonic splicing regulators, ESRs) and/or in introns (intronic splicing regulators, ISRs) are required for an accurate splice site selection. These determine the interaction of additional proteins designated splicing factors, which bind to them and in this way direct the splicing machinery to the correct splice sites (see Figure 1) (Matlin et al., 2005; Nilsen & Graveley, 2010; Reddy, 2007; Wachter et al., 2012).

One of the main conserved families of such splicing factors is the SR (serine/arginine-rich) family of RNA-binding proteins, which are essential for the execution and regulation of pre-mRNA splicing, playing vital roles in both constitutive and alternative splicing. SR proteins are present in metazoans and plants and share a characteristic structural organization, consisting of one or two N-terminal RNA-recognition motifs (RRMs) that bind to regulatory sequences in the pre-mRNA, and a C-terminal arginine/serine-rich (RS) domain that not only promotes protein-protein interactions with other components of the splicing machinery but also promotes pre-spliceosome assembly by contacting directly with the pre-mRNA via the branch-point and the 5' splice site (Barta et al., 2010; Hertel & Graveley, 2005; Shen & Green, 2004; Shen et al., 2004; reviewed in Duque, 2011; Ram & Ast, 2007; Reddy & Shad Ali, 2011). By being able to bind the RNA and interact with spliceosomal components directing them to the correct splice sites, SR proteins are important players in alternative splicing, determining splice site selection under different conditions in a concentration-dependent manner (Graveley, 2000; Manley & Tacke, 1996). In animals, these RNA-binding proteins have also been implicated in mRNA transport (Huang & Steitz, 2001), mRNA stability and mRNA nonsense-mediated decay (NMD) (Zhang & Krainer, 2004) and translation (Sanford et al., 2004). In plants, a multitude of SR proteins has been identified in Arabidopsis (Lorkovic & Barta, 2002), rice (Iida & Go, 2006; Isshiki et al., 2006), Brachypodium (International Brachypodium, 2010), maize and sorghum (Rauch et al., 2014). Based on the recent updated definition for plant SR proteins, the rice SR family comprises of 22 members while Arabidopsis has 18 (Barta et al., 2010).

2. The Arabidopsis SR45 splicing factor

The Arabidopsis SR45 is a plant specific SR-related protein. It functions as an essential splicing factor, being able to complement in vitro an animal splicing extract deficient in SR proteins (Ali et al., 2007).

Since it displays a highly atypical SR protein structure, i.e. one RRM flanked by two distinct RS domains (Figure 3), and due to the recent revision of the plant SR family classification criteria (Barta et al., 2010), SR45 has been excluded from this family of splicing regulators. The two RS domains of SR45 present different complexity levels with the N-terminal domain (RS1) being rich in serine and arginine-serine repeats (S/RS) and the more complex C-terminal domain (RS2) containing arginine-serine repeats (RS) and serine-proline-x-arginine motifs (SPXR) (Figure 3) (Zhang & Mount, 2009).



Figure 3. Domain organization of the Arabidopsis SR45 protein

Schematic diagram showing the domain organization of the SR45 protein, including the N-terminal RS1 domain, the middle RRM and the C-terminal RS2 domain. RRM (RNA Recognition Motif), RS1 and RS2 (Arginine/Serine rich domains 1 and 2).

The *SR45* gene (At1g16610) comprises twelve exons and is reported to generate three splice variants. In 2007, a comprehensive analysis of the Arabidopsis SR protein gene family revealed two SR45 isoforms that differ by a 21-nucleotide sequence present in isoform 1 (*SR45.1*) and missing in isoform 2 (*SR45.2*) due to an alternative 3' splice site event occurring in intron 6 (Palusa et al., 2007). The proteins encoded by these isoforms are very similar, with *SR45.1* including an additional 7-amino acid segment (T²¹⁸S²¹⁹P²²⁰Q²²¹R²²²K²²³T²²⁴). The third splice variant, *SR45.3*, has been annotated in The Arabidopsis Information Resource (TAIR) database (<http://www.arabidopsis.org>). This transcript arises from selection of an alternative 5' splice site in intron 9 leading to the inclusion of a 33-nt sequence that is absent from the other two splice variants sequence, encoding a protein with 11 additional amino acids. All the alternatively spliced segments are in frame, so none of the SR45 splice variants give rise to a truncated form of the protein.

The SR45 splicing factor has been extensively characterized in plants. It was first isolated in a Yeast-2-Hybrid screen where it was shown to interact with the U1 snRNP 70K protein, known to function in 5' SS selection, as well as with the SCL33 SR splicing factor (Golovkin & Reddy, 1999). In 2012, Day et al. provided evidence that besides U1-70K, SR45 also interacts with another spliceosomal protein, U2AF³⁵, responsible for 3'SS selection, and directly binds SR30 splicing factor. Their results suggest that SR45 modulates 5' and 3' site selection and may bridge the 5' and 3' components of the spliceosome.

Expression of SR45 fused with GFP in cultured onion and tobacco cells and transgenic Arabidopsis plants has shown that SR45 is localized to the nucleus, diffusely distributed in the nucleoplasm or concentrated in speckles of different sizes and shapes (Ali et al., 2003). These speckles correspond to

interchromatin granule clusters that serve as storage/re-cycling sites for splicing factors (Misteli, 2000; Misteli et al., 1997) and deliver them to nearby active sites of transcription (Misteli & Spector, 1998). A loss-of-function mutant for SR45, *sr45-1*, was first reported by Ali et al. (2007), and its characterization showed that this splicing factor plays an important role in normal plant development. The authors found that depletion of SR45 levels leads to pleiotropic developmental phenotypes, such as narrow leaves, altered number of petals and stamens, delayed root growth and late flowering. In addition, the alternative splicing pattern of five SR genes was changed in the *sr45-1* mutant, indicating that the observed phenotypes could be due to altered levels of these SR protein isoforms, which in turn would regulate the splicing of other transcripts (Ali et al., 2007). In 2012, Ausin et al. reported that *sr45-1* mutants exhibit impaired DNA methylation establishment capacity and therefore are unable to methylate the *FWA* gene, a homeodomain-containing transcription factor that controls flowering. This could in part explain the flowering phenotype of the *sr45-1* mutant. Interestingly, Zhang and Mount (2009) demonstrated that two of the alternatively-spliced SR45 isoforms (SR45.1 and SR45.2) have distinct biological functions. The authors generated stable transgenic lines expressing *SR45.1-GFP* and *SR45.2-GFP* constructs in the *sr45-1* mutant background and observed that the SR45.1-GFP rescues the flower petal phenotype but not the root growth, while the plants overexpressing the second splice variant only complement the root growth phenotype. These results indicate that SR45.1 has a major role in flower petal development and SR45.2 in root growth. Very recently, Zhang et al. (2014) showed that a single phosphorylation of T²¹⁸ in the 21nt-sequence present in SR45.1 but absent from SR45.2, is key to the distinct function of the former isoform in petal formation. The authors also revealed that phosphorylation of this amino acid is required for the function of SR45.1 in promoting splicing of its direct target, the *SR30* mRNA.

In 2010, Carvalho et al. presented evidence that the SR45 splicing factor acts as negative regulator of sugar and abscisic acid (ABA) signaling during early seedling development. The authors observed that the *sr45-1* mutant is hypersensitive to glucose, displaying impaired cotyledon greening and expansion when grown under the presence of this sugar. The mutant also exhibits hypersensitivity to the stress hormone ABA along with enhanced glucose-induced ABA accumulation, which is responsible for the observed glucose oversensitivity. The same authors have subsequently found that disruption of the *Hexokinase (HXK) 1* gene, encoding a conserved glucose sensor, does not suppress the glucose phenotype of the *sr45-1* mutant, indicating that the mode of action of SR45 in the sugar signalling pathway is independent of this sensor (Carvalho et al., submitted). Interestingly, SR45 control of sugar responses largely depends on the serine/threonine SnRK1.1 kinase, since disruption of the *SnRK1.1* gene leads to a significant rescue of the *sr45-1* glucose phenotype (Carvalho et al., submitted). Moreover, despite unchanged transcript levels, *sr45-1* leaves contain significantly higher

amounts of SnRK1.1 in the presence of glucose as a result of reduced proteasomal degradation of this protein. The SnRK1.1 kinase has been previously described as a sensor and signaling component of stress-associated energy deprivation (Baena-González et al., 2007) and is involved in sugar and ABA signaling (Jossier et al., 2009). An inositol polyphosphate 5-phosphatase, 5PTase13, interacts with and stabilizes SnRK1.1 in vitro (Ananieva et al., 2008) and is proposed to act in a nutrient-dependent manner. Under limited sugar supply, 5PTase13 binds SnRK1.1 protecting it from proteasomal degradation. Unpublished data by Carvalho et al. show that the splicing pattern of this phosphatase gene is markedly altered in the *sr45-1* mutant, suggesting that SR45 regulates SnRK1.1 levels in response to sugars by modulating alternative splicing of the *5PTase13* gene.

3. Inositol polyphosphate 5-phosphatases

In order to adapt and survive to a constantly changing environment, living organisms evolved a series of receptors and signaling pathways, responsible for transmitting information within the cell. External stimuli arrive at the cell surface, usually in the form of a chemical signal, and are perceived by membrane-bound receptors that convert them into intracellular secondary messengers, which then amplify and relay the signals to target molecules (Berridge, 1993). Many organisms use the inositol triphosphate (Ins(1,4,5)P₃) signaling pathway (Figure 4). This pathway is initiated by signal-induced activation of phospholipase C (PLC), which then cleaves the phosphatidylinositide (4,5) biphosphate (PtdIns(4,5)P₂) substrate producing the secondary messenger Ins(1,4,5)P₃ (Figure 4). This secondary messenger stimulates a transient release of calcium (Ca²⁺) from intracellular stores that will then trigger a series of downstream biological responses (Figure 4). The signal induced by Ins(1,4,5)P₃ ends with its consecutive dephosphorylation into free inositol. The first of these dephosphorylation reactions is catalyzed by a group of specific inositol phosphatases referred to as inositol polyphosphate 5-phosphatases (5PTases) (Figure 4). These enzymes remove specifically the 5-position phosphate from the inositol ring of Ins(1,4,5)P₃, as well as of other inositol-containing secondary messengers, such as the water-soluble inositol polyphosphates Ins(1,3,4,5)P₄ and/or the phosphoinositides PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, all important signaling molecules in eukaryotic organisms (Majerus et al., 1999). Phosphoinositides are ubiquitous phospholipid constituents of cell membranes, and their phosphorylation generates a collection of signaling molecules with discrete functions. By regulating the levels of specific phosphoinositides, 5PTases allow the regulation of several cellular processes, such as protein trafficking, phagocytosis and synaptic vesicle recycling, with their gain or loss of function having been implicated in human disease (reviewed in Ooms et al., 2009).

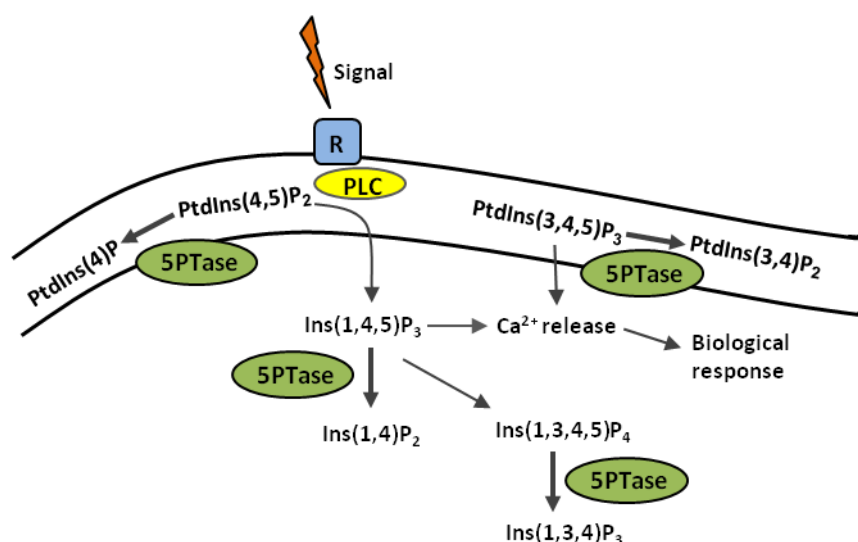


Figure 4. Inositol signaling and its termination via 5PTases

Termination of signaling in mammals can occur by hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ and three other inositol-containing secondary messengers by 5PTases. **R**, putative receptor; **PLC**, phospholipase C; **PtdIns(4,5) P_2** , phosphatidylinositide (4,5) biphosphate; **PtdIns(3,4,5) P_3** , phosphatidylinositide (3,4,5) triphosphate; **Ins(1,4,5) P_3** , inositol (1,4,5) triphosphate; **Ins(1,3,4,5) P_4** , inositol tetrakisphosphate. Adapted from Berdy et al. (2001).

5PTases belong to the large Exonuclease/Endonuclease/Phosphatase (EEP) superfamily (Marchler-Bauer et al., 2013) and are characterized by the presence of a conserved 5PTase catalytic domain of around 350 amino acid residues, in which there are two conserved catalytic motifs that are essential for the 5PTase activity (Whisstock et al., 2002). There are four known substrates for 5PTases: the water-soluble $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, and the lipids $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$. Majerus et al. (1999) have classified mammalian 5PTases according to their substrate specificity into four groups. **Type I** 5PTases hydrolyze the phosphate from $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ and are the most active of all 5PTases toward water-soluble inositol polyphosphates, while **Type II** can hydrolyze the phosphate of both water-soluble inositol polyphosphates and lipid phosphoinositides. Whereas **Type III** 5PTases can hydrolyze phosphates from $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (substrates with a 3-position phosphate group), **Type IV** 5PTases can only specifically dephosphorylate $\text{PtdIns}(3,4,5)\text{P}_3$.

Based on sequence homology to known 5PTases of other organisms, Berdy et al. (2001) predicted that there are at least 15 genes in *Arabidopsis thaliana* encoding conserved 5PTases. These authors identified two classes of plant 5PTases according to their phylogenetic relationship: **group A** comprises 11 smaller 5PTases (5PTase1 to 5PTase11) that only contain the conserved 5PTase catalytic domain; while **Group B** contains four larger 5PTases (FRA3 and 5PTase12 to 5PTase14) that in addition to the 5PTase catalytic domain also incorporate a WD-repeat (or WD40) domain, with 5 to 6 WD-repeats (Zhong & Ye, 2004), a novel combination only present in plant 5PTases (Figure 5). The

WD-repeat is a conserved structural motif that adopts a beta propeller structure thought to create a stable platform to which several proteins can bind to reversibly, hence allowing sequential and/or simultaneous interactions with different protein sets (Smith et al., 1999; reviewed in Stirnimann et al., 2010).

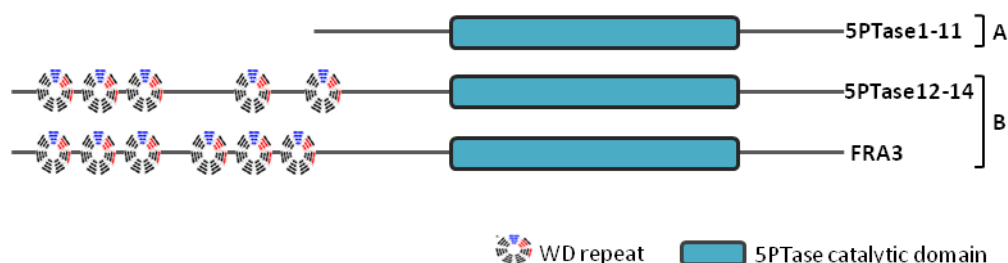


Figure 5. Schematic representation of the domain organization of Arabidopsis 5PTases

Both classes of 5PTases have a conserved 5PTase catalytic domain. Class A (5PTase1-11) only has this catalytic domain, while class B (5PTase12-14 and FRA3) also includes a WD-repeat domain (novel combination only present in plant 5PTases).

In vitro, when expressed as recombinant proteins, most of the Arabidopsis 5PTases hydrolyze both InsP and PtdInsP substrates. The exceptions are 5PTase11 (Ercetin & Gillasp, 2004) and 5PTase6/CVP2 (Carland & Nelson, 2009), which can only hydrolyze PtdInsP, and 5PTase12 and 5PTase13, capable of only hydrolyzing Ins(1,4,5)P₃ (Zhong & Ye, 2004).

In plants, knowledge of the function of 5PTases is still very limited, but genetic approaches have allowed the functional characterization of several 5PTases in Arabidopsis, revealing diverse roles of various aspects of plant growth and development as well as in stress responses. For instance, a mutation in the *FRAGILE FIBER3* (*FRA3*) 5PTase gene in Arabidopsis causes an accentuated reduction in secondary wall thickness and altered actin organization in fiber cells resulting in a decrease in stem strength (Zhong et al., 2004), while mutants for the *COTYLEDON VASCULAR PATTERN 2* (*CVP2*)/5PTase6 gene exhibit an abnormal venation pattern in their leaves and cotyledons, as well as ABA hypersensitivity (Carland & Nelson, 2004). The MORPHOGENESIS OF ROOT HAIR 3 (*MRH3*)/5PTase5 protein is involved in root hair development, being required for restricting both the size of the root-hair initiation site and the width of the root hairs during the transition to tip growth (Jones et al., 2006). In addition, the 5PTase1, 5PTase2 and 5PTase11 proteins have been shown to be required for growth regulation in seedlings (Ercetin et al., 2008; Gunesequera et al., 2007). Loss of function mutants for 5PTase11 display a delay in germination and a decrease in hypocotyl growth when grown in the dark (Ercetin et al., 2008) while, in the same conditions, mutants for 5PTase1 and 5PTase2 germinate faster and have longer hypocotyls than wild-type plants (Gunesequera et al., 2007). Interestingly, the latter study showed that the 5ptase1 and 5ptase2 mutants are also

hypersensitive to exogenous ABA. Recent studies have implicated 5PTase7 and 5PTase9 phosphatases in plant responses to salt stress, with knockout mutants for each of these genes showing increased salt sensitivity associated with a reduction in the production of reactive oxygen species (ROS) and altered stress-responsive gene expression (Golani et al., 2013; Kaye et al., 2011). In 2012, Wang et al., showed that a mutation in the *5PTase12* gene is responsible for precocious pollen germination. Interestingly, loss of function of *5PTase13* enhances this phenotype, with the *5ptase12 5ptase13* double mutants presenting an even higher precocious pollen germination rate, as well as higher levels of $\text{Ins}(1,4,5)\text{P}_3/\text{Ca}^{2+}$. These results suggested that control of $\text{Ins}(1,4,5)\text{P}_3/\text{Ca}^{2+}$ by 5PTases is crucial for maintaining pollen dormancy. Finally, it is worth noting that many of the reported 5PTases (5PTase1, 5PTase2, CVP2, 5PTase11 and 5PTase13) are implicated in ABA signaling (Ananieva et al., 2008; Burnette et al., 2003; Carland & Nelson, 2004; Ercetin & Gillaspay, 2004; Gunesequera et al., 2007; Lin et al., 2005; Sanchez & Chua, 2001; Zhong & Ye, 2004).

3.1. Arabidopsis 5PTase13

As mentioned above, inositol polyphosphate 5-phosphatase 13 (5PTase13 - At1g05630), belongs to the group B of 5PTases, harboring both a catalytic domain and a WD-repeat domain (Zhong & Ye, 2004) (Figure 6). Although it presents the highest sequence similarity to type II 5PTases in animals, biochemically it belongs to Type I, as it exhibits phosphatase activity specifically toward the water-soluble $\text{Ins}(1,4,5)\text{P}_3$ (Zhong & Ye, 2004).

Functional characterization studies on 5PTase13 showed that this protein plays important roles in plant development as well as in hormonal and light signaling pathways. For instance, Lin et al. (2005) have reported the involvement of 5PTase13 in the regulation of auxin signaling and homeostasis, with *5PTase13* loss-of-function mutants presenting cotyledon vein development and patterning defects that are completely rescued by exogenous auxin. In addition, 5PTase13 has also been implicated in auxin transport (Wang et al., 2009). PIN proteins are essential for auxin distribution and root gravitropism and Wang et al. (2009) observed that *5PTase13* knockout mutants display enhanced sensitivity to gravistimulation, which is related to an expanded PIN2 expression in mutant roots as well as altered vesicle transport. In 2012, the same authors showed that 5PTase13 function is also important in pollen dormancy maintenance, as previously mentioned. Chen et al. (2008), have reported that *5PTase13* is also involved in blue light responses in *Arabidopsis thaliana*. Disruption of the *5PTase13* gene results in shortened hypocotyls and expanded cotyledons under blue light, accompanied by increased calcium levels, with epistatic analyses showing that *5PTase13* functions downstream of *PHOTOTROPIN 1* (*PHOT1*), a blue light receptor controlling plant phototropism. Although unable to reproduce the vascular pattern phenotype described by Lin et al. (2005),

Ananieva et al. (2008) have implicated 5PTase13 in nutrient/energy signaling, showing that the WD40 repeat region of *5PTase13* interacts with the SnRK1.1 energy sensor. When compared with wild type seedlings, the *5ptase13-1* and *5ptase13-2* loss-of-function mutants display increased SnRK1.1 activity under no nutrient conditions. The opposite was observed when seedlings were grown under low nutrients or in the presence of 6% glucose, suggesting that 5PTase13 acts as a negative regulator of SnRK1.1 activity in the absence of nutrients, and as a positive regulator under low nutrients or 6% glucose. These mutants also display reduced root growth under no nutrient or low nutrient conditions, while germination assays indicate them to be insensitive to high sugar concentrations as well as to ABA (Ananieva et al., 2008). In 2004, Zhong and Ye showed that *5PTase13* was significantly induced by ABA and also wounding. Finally, localization studies show that GFP-tagged 5PTase13 is localized to the nucleus (Ananieva et al., 2008).

4. Objectives

This present work had as main goals:

- 1) To investigate whether the production of the two *5PTase13* splice variants have *in vivo* functional impact in plant sugar and ABA responses;
- 2) To assess potential functional interactions between the two 5PTase13 isoforms and SR45, a negative regulator of sugar and ABA signaling;
- 3) To study the subcellular localization of the two *5PTase13* isoforms.

II. MATERIALS AND METHODS

1. Plant materials and growth conditions

Arabidopsis thaliana (L.) Heyhn, ecotype Columbia-0 (Col-0) was used as the wild type in all experiments. A homozygous line for the *sr45-1* T-DNA insertion mutant (SALK_004132) was previously isolated by Carvalho et al. (2010) and seeds homozygous for T-DNA insertions within the *5PTase13* gene, *5ptase13-1* (SAIL_350_FO1) and *5ptase13-2* (SALK_081991) were kindly provided by Dr. Glenda Gillaspay from Virginia Tech, USA (Ananieva et al., 2008).

Seeds were surface-sterilized for 10 min in 50% (v/v) bleach and 0.028% (v/v) Tween 20 under continuous shaking and then washed three times in sterile water. Following stratification for three days in the dark at 4°C (to break dormancy), seeds were sown in Petri dishes containing Murashige & Skoog (1 X MS) salts (Duchefa Biochemie) supplemented with 0.5 mM myo-inositol and 2.5 mM MES, adjusted to pH 5.7 with 1 M KOH and solidified by 0.8% agar. The plates were then placed in a controlled environment reach-in chamber under long-day conditions, 16 h light (100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, cool white fluorescent light), 22°C / 8 h dark, 18°C, and 60% relative humidity. After 2-3 weeks, plants were transferred to soil in individual pots and placed in a growth chamber under the conditions described above.

2. Isolation of T-DNA insertion mutants

The T-DNA insertion lines SALK_083676, SALK_140382, GABI_028H03 and GABI_187B05 in the *5PTase13* (At1g05630) gene (Table 1) were identified from the Arabidopsis insertion mutant library “TDNA-express: Arabidopsis gene mapping tool” (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and purchased from the Nottingham Arabidopsis Stock Centre (NASC). Seeds from these insertion lines were surface sterilized as described above and selected for kanamycin (SALK lines) or sulfadiazine (GABI lines) resistance by plating seeds on 1 X MS medium supplemented with 50 $\mu\text{g/mL}$ of kanamycin (K4378; Sigma) or 7.5 $\mu\text{g/mL}$ sulfadiazine (S8626; Sigma), respectively. As a precaution, in case of silencing of kanamycin/sulfadiazine resistance, some seeds were plated on 1 X MS medium alone. As none of the lines exhibited silencing of the marker gene, only the seedlings that grew on the specific resistance antibiotic were selected and transferred to soil.

The progeny of the mutant lines was genotyped by PCR using primers specific to the T-DNA vector and the *5PTase13* gene (Table 1). All PCR products were separated and visualized on 1% agarose gels. To confirm the exact insertion location, PCR products from homozygous mutant were sequenced (AB DNA Sequencer 3130xl).

Table 1. T-DNA insertion lines and corresponding gene-specific primers used for mutant line genotyping

T-DNA insertion lines for the Arabidopsis *5PTase13* gene obtained from the SALK and GABI-Kat collections, as well as antibiotic resistance marker and sequences of the primers used.

Gene name	Locus ID	Line	Antibiotic resistance	Primers
<i>5PTase13</i>	At1g05630	SALK_083676	Kan	F2: 5'-TCGAAGAAGAAGACGAAGAGGC-3' R4: 5'- ATCTCCAGTCTGCGTCTCCC-3'
		SALK_140382	Kan	F: 5'-CTCTCTCTCTCCACCACC-3' R2: 5'- CAGGACCACGATGAGCTAGCC-3'
		GABI_028H03	Sulf	F: 5'-GTGGTCATGTTCTGAAGGAGG-3' R: 5'-CACATACCATCCTCGTACTCC-3'
		GABI_187B05	Sulf	F: 5'-GTGGTCATGTTCTGAAGGAGG-3' R: 5'-CACATACCATCCTCGTACTCC-3'
T-DNA segment			LBd: 5'-CCTGATAGACGGTTTTTCGCCC-3'	

Kan, Kanamycin; Sulf, Sulfadiazine

3. Phenotypical analyses

Plants of different genotypes were sown and grown simultaneously under identical conditions. Seeds from fully matured siliques of dehydrated plants of the same age were collected and stored in the dark at room temperature. All assays were performed using seeds from comparable lots stored for 1-24 months.

For seed germination assays, seeds were sterilized (as described above) and water imbibed in the dark for 3 days at 4°C. After stratification, 80-100 seeds of each genotype were sown in triplicate in Petri dishes containing 0.5 X MS medium (MS salts, 2.5 mM MES pH 5.7, 0.5 mM myo-inositol and 0.8% agar), supplemented or not with the appropriate concentrations of D-glucose (Sigma) or ABA (mixed isomers, A1049; Sigma). ABA was dissolved in 100% ethanol and added to the cooled, sterile medium at the desired concentration. Plates were then placed in a controlled environment reach-in chamber under long-day photoperiod (16 h light, 22°C / 8 h dark, 18°C) and 60% relative humidity under standard light (100 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) or low light (40 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) conditions.

Germination (defined as the protrusion of the radicle from the seed coat), cotyledon greening and cotyledon expansion were scored every day after transfer to the growth chamber, with cotyledon greening and expansion rates being calculated over the total of germinated seeds. Average percentages were calculated with SEs of the triplicates.

For root growth assays, approximately 40 seeds per genotype were sterilized and sown in duplicate on square plates containing 0.5 X MS medium (MS salts, 2.5 mM MES pH 5.7, 0.5 mM myo-inositol and 0.8% agar). Plates were kept in the dark, vertically oriented, for 3 days at 4°C for stratification,

being transferred after that period to a controlled environment reach-in chamber with the same settings described above under low light ($40 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) conditions. Root tip positions were scored every 2 days. Primary root length was measured on scanned images using ImageJ (<http://rsbweb.nih.gov/ij>). All assays were repeated at least three times with similar results.

4. Generation of transgenic lines

Constructs previously cloned in the lab containing the *5PTase13.1*- or *5PTase13.2*-GFP-tagged wild-type coding sequence under the control of the 35S promoter were introduced into the *Agrobacterium tumefaciens* strain EHA105 for floral dip transformation (Clough & Bent, 1998) of *sr45-1* homozygous plants (*sr45-1/35S:5PTase13*). Wild-type (Col-0) and mutant (*sr45-1*) plants were also transformed with the empty GFP vector (Col-0 \emptyset and *sr45-1* \emptyset). T1 transformants were selected in BASTA-containing medium, grown to maturity and selfed. At least six independent transgenic lines of each construct were selected and all phenotypical analyses were conducted at the T2 generation.

5. RNA extraction and RT-PCR analyses

Total RNA was extracted using the innuPREP Plant RNA kit (Analytik Jena), according to the manufacturer's protocol, and all RNA samples were digested with DNaseI (Promega) and phenol-chloroform purified. Reverse transcription was then performed on 1 μg of total RNA with M-MLV reverse transcriptase (Promega) as instructed by the manufacturer.

For RNA analysis of homozygous mutants, as well as of *sr45-1/35S:5PTase13* transgenic plants, total RNA was extracted from 2 week-old plantlets.

For the tissue-specific expression and splicing patterns, and expression analysis at the different developmental stages, plant material was harvested from seedlings/plantlets at different timepoints after stratification (7, 14 and 21 days) or from one individual 8-week old plant. Total RNA from siliques, stems, senescent leaves and roots was extracted using the innuPREP Plant RNA kit mentioned above, whereas the RNA from the remaining organs (young leaves, cauline leaves, adult leaves, flowers, seedlings and plantlets) was extracted with TriReagent (SIGMA) according to the manufacturer's instructions. All RNA was DNase-treated and phenol-chloroform purified before being reverse transcribed, as described above.

The *5PTase13* gene-specific primer pairs used are listed in Table 2. For PCR amplification, 35 cycles were performed, except for the housekeeping genes β -ACTIN (*ACT*) and CYCLOPHILIN (*ROC1*) (Table 2), which were detected after 23 and 28 cycles, respectively. All PCR products were separated on 1%

agarose gels and quantification of the amplified bands was performed with ImageJ (<http://rsbweb.nih.gov/ij>).

Table 2. Gene-specific primers used in the analysis of the expression and splicing patterns of the Arabidopsis *5PTase13* gene

Primers specific for the *5PTase13* gene (annealing in exonic sequences) as well as for two housekeeping genes, *β-ACTIN* (*ACT*) and *CYCLOPHILIN* (*ROC1*).

Gene Name	Locus ID	Primers
<i>5PTase13</i>	At1g05630	F1: 5'-AGAAACGGTTGGAAGG-3' F2: 5'-TCGAAGAAGAAGACGAAGAGGC-3' F3: 5'-AAGGAACTTTATATGCCAGAC-3' R1: 5'-ATAAGCTGAAGTTGAGACACC-3' R2: 5'-CAGGACCACGATGAGCTAGCC-3' R3: 5'-CACATGGAAGTCTGCAACATC-3' R4: 5'-ATCTCCAGTCTGCGTCTCC-3'
<i>ACT</i>	At3g18780	F: 5'-TTTGCAGGAGATGATGCTCCC-3' R: 5'-GTCTTTGAGGTTTCCATCTCC-3'
<i>ROC1</i>	At4g38740	F: 5'-GTCTGATAGAGATCTCACGT-3' R: 5'-AATCGGCAACAACAACAGGC-3'

6. Confocal laser scanning microscopy

Confocal images were obtained with an LSM 510 laser-scanning microscope equipped with a Meta detector (Zeiss). For cell wall staining, roots were incubated in 10 µg/mL iodide propidium for 10 min. Excitation wavelengths used to detect fluorescence were 488 nm for GFP and 543 nm for iodide propidium. Emitted fluorescence was monitored at detection wavelengths between 500 and 550 nm for GFP and longer than 560 nm for iodide propidium.

III. RESULTS

1. Expression and splicing pattern of the *5PTase13* gene in Arabidopsis

The structure of the Arabidopsis *5PTase13* gene (At1g05630) is shown in Figure 6. The current genome annotation (TAIR10; www.arabidopsis.org) indicates that this gene generates two distinct splice variants due to an intron retention event. The first splice variant (*5PTase13.1*) results from inclusion of the 102-nt long intron, which is spliced out from the second splice variant (*5PTase13.2*) (Figure 6). As this alternatively-spliced fragment is in frame, the two predicted *5PTase13* protein isoforms differ only in 34 amino acids. Although no recognizable protein domain is included in this region, it falls within the catalytic domain of the phosphatase. Both splice forms exhibit the five WD40 repeats, the *5PTase* catalytic domain and the two conserved motifs in the same position (Figure 6). However, the longer protein isoform (*5PTase13.1*) contains one extra N-myristoylation site (Figure 6).

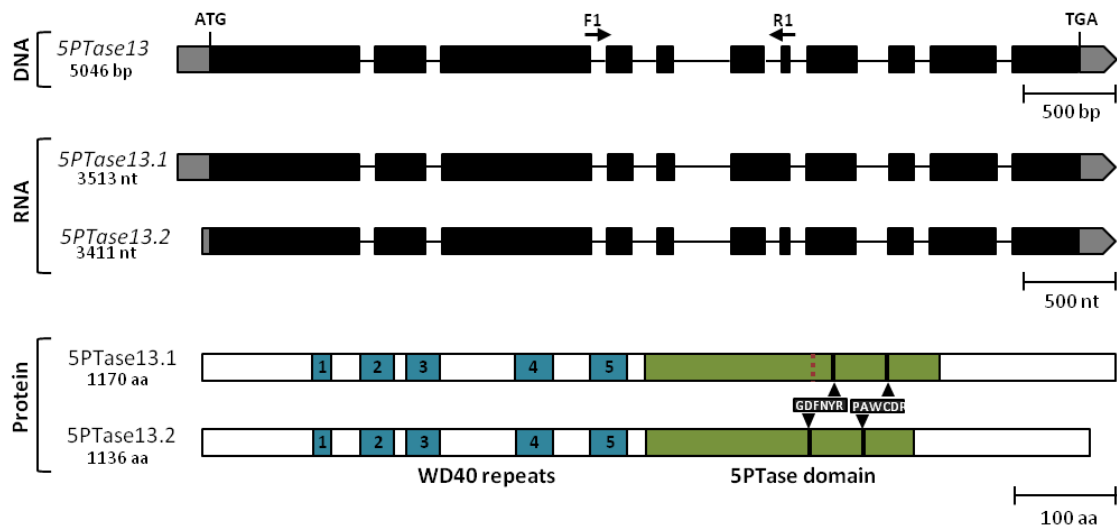


Figure 6. Structure of the Arabidopsis *5PTase13* gene and its alternative transcripts, as well as domain organization of their encoded protein isoforms

Exon/intron organization of the *5PTase13* gene, where the boxes represent exons with UTRs in grey and lines represent introns. The arrows indicate the location of *5ptase13*-specific primers used in the RT-PCR shown in Figure 7. The structure of the two splice variants (*5PTase13.1* and *5PTase13.2*) and of the corresponding predicted protein isoforms is also shown — the WD40 repeats and the *5PTase* domain are represented by the blue and green boxes, respectively, the two black bars within the *5PTase* domain mark the position of two conserved motifs (Zhong & Ye, 2004) and the dashed pink bar indicates the N-myristoylation site that is missing in the *5PTase13.2* protein isoform.

To evaluate the *5PTase13* development- and tissue-specific expression pattern in wild-type (Col-0) plants, RT-PCR analysis was conducted. Primers flanking the alternatively-spliced segment were

designed for the detection of both splice variants simultaneously (F1 and R1, Table 2, Figure 6). The amplified bands were then quantified and the ratio between *5PTase13.1*/*5PTase13.2* (SV1/SV2) calculated. Our results show that both *5PTase13* transcripts are ubiquitously expressed in Arabidopsis, being present in seedlings and different aged plantlets, as well as in different aerial organs (leaves of different ages, stems, flowers and siliques) and roots, with the shorter splice variant, *5PTase13.2*, being by far the most abundant (Figure 7). Interestingly, both transcripts are less expressed in young (seven-day-old) seedlings. The ratio between the transcript levels of *5PTase13.1* and *5PTase13.2* is very similar in all organs and in all different aged seedlings and plantlets except for 14 day-old plantlets where expression of *5PTase13.1* is markedly higher when compared with 7-day-old seedlings and 21-day-old plantlets, as well as with all the other analyzed organs (Figure 7).

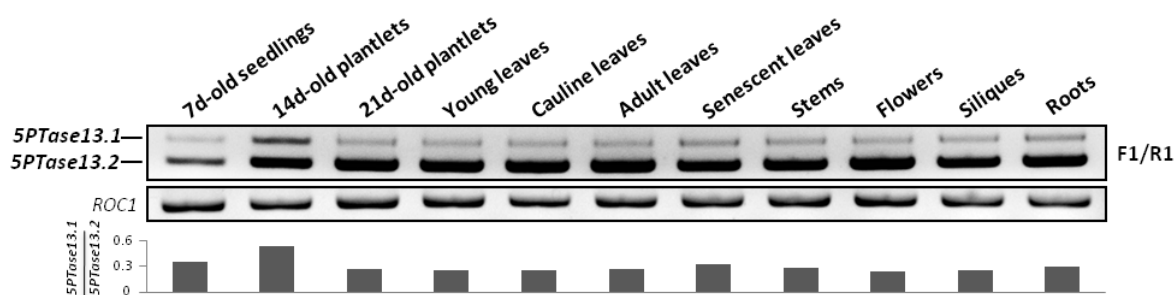


Figure 7. Expression and splicing pattern of the Arabidopsis *5PTase13* gene

RT-PCR analysis of the transcript levels of the two *5PTase13* splice variants, *5PTase13.1* and *5PTase13.2*, at different developmental stages and in different organs of Arabidopsis wild-type (Col-0) plants. The location of the F1 and R1 primers used is shown in Figure 6. Expression of the *CYCLOPHILIN* (*ROC1*) gene was used as a loading control. Bands were quantified and the histogram shows *5PTase13.1*/*5PTase13.2* ratios for each sample.

2. Isolation of a null *5PTase13* loss-of-function mutant

There are very few plant genes for which the production of multiple splice isoforms has been reported to have a clear *in vivo* functional impact. Since the *5PTase13* gene undergoes alternative splicing, one of our goals was to investigate the potential function of the two *5PTase13* isoforms by means of their individual expression in the *5PTase13* null background. Although there were already two previously isolated mutants for this gene – *5ptase13-1* and *5ptase13-2* (Ananieva et al., 2008; Lin et al., 2005) (Figure 8a), the lines kindly provided by Dr. Glenda Gillaspay from Virginia Tech, USA, were knockdown mutants. As seen in Figure 8b, RT-PCR analyses show that *5PTase13* expression in each mutant is not completely abolished. This could explain why we had difficulties in reproducing most of the phenotypes reported by Ananieva et al. (2008) (data not shown) and underscored the

need for the isolation of a true genetic null *5PTase13* mutant. To this end, after checking the available options at “TDNA-express: Arabidopsis gene mapping tool” (<http://signal.salk.edu/cgi-bin/tdnaexpress>), several lines carrying a T-DNA insertion within the *5PTase13* gene were obtained from the SALK and GABI-Kat collections (Table 1). A schematic representation of the *5PTase13* gene and the location of all screened T-DNA insertion lines is shown in Figure 9a.

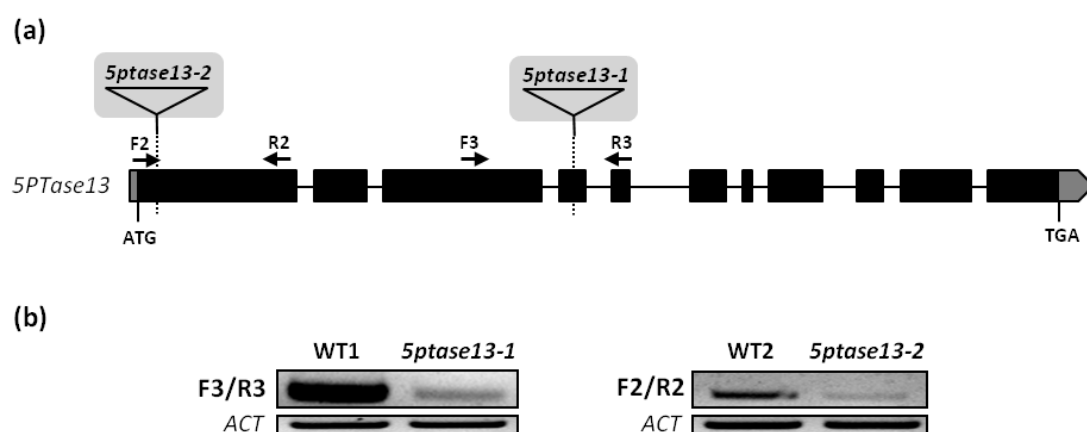


Figure 8. Molecular characterization of two T-DNA insertion mutants for the *5PTase13* gene

(a) Schematic representation of the *5PTase13* gene showing the T-DNA insertion sites (triangles) in the *5ptase13-1* and *5ptase13-2* mutants described by Ananieva et al. (2008). Boxes indicate exons with UTRs in grey, lines between boxes represent introns, and arrows indicate the location of the F2, R2, F3 and R3 *5ptase13*-specific primers. **(b)** RT-PCR analysis of *5PTase13* gene expression in Col-0 wild-type (WT1 and WT2) and mutant (*5ptase13-1* and *5ptase13-2*) plants. The location of the F2, R2, F3 and R3 primers used is shown in (a). Expression of the *ACTIN* (*ACT*) gene was used as a loading control.

The obtained mutant lines were first screened for resistance to the specific selection marker for T-DNA insertions – kanamycin for SALK lines or sulfadiazine for GABI-Kat lines (Table 1). All resistant plants were then genotyped using gene-specific primers flanking the insertion site reported in the SALK database and the T-DNA-specific primer LBd that anneals at the end of the left border (LB) of the T-DNA (Table 1). This screen allowed the isolation of a homozygous mutant line for SALK_083676 containing the T-DNA insertion in exon 1, which was named *5ptase13-3* (Figure 9). Regarding the other mutant lines, GABI_187B0585, GABI_028H03 and SALK_140382 (Figure 9a), a total of 60, 85 and 19 plants, respectively, were genotyped but no plants homozygous for the insertion were retrieved.

PCR-based genotyping using gene-specific primers flanking the insertion in SALK_083676 (Figure 9b, Tables 1 and 2) amplified the expected size product for Col-0 (WT3), but not for the homozygous line, confirming gene disruption in the *5PTase13* gene (Figure 9c). On the contrary, PCR using the LBd primer only amplified a product in the mutant line and not in the corresponding Col-0 wild type

(WT3), confirming the presence of the insertion (Figure 9c). The obtained fragment was then sequenced to verify the exact insertion site.

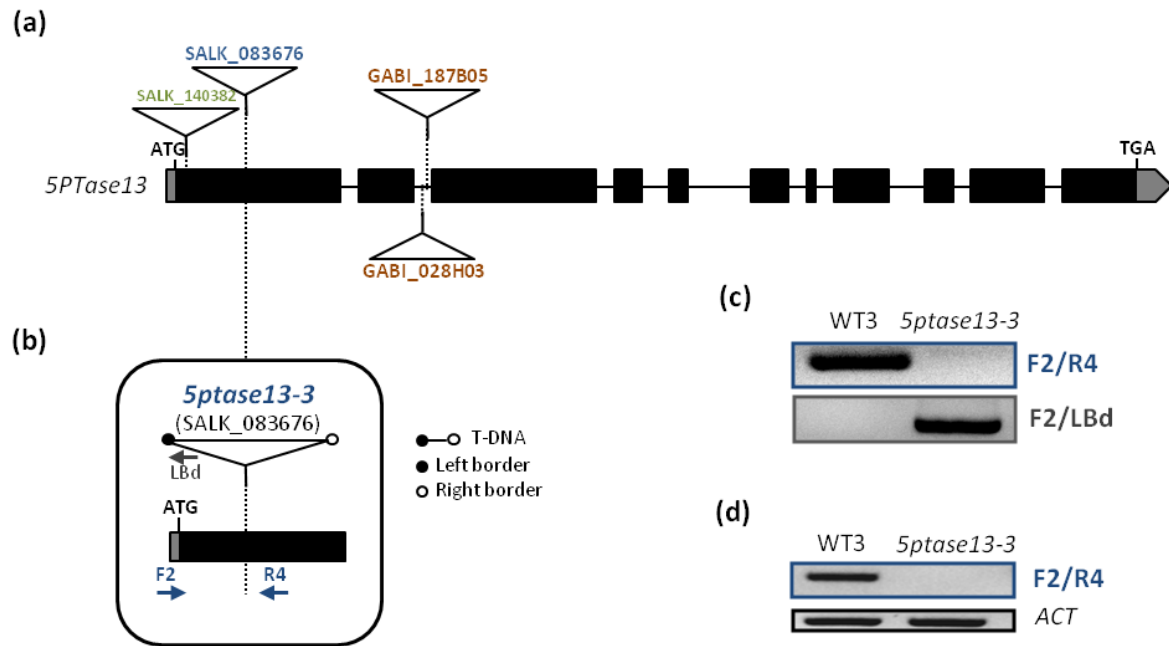


Figure 9. Isolation of the *5ptase13-3* null mutant

(a) Exon/intron organization of the *5PTase13* gene (boxes represent exons with UTRs in grey and lines represent introns) and location of the T-DNA insertion sites (triangles) in the mutant lines obtained from the SALK and GABI-Kat collections. **(b)** Schematic representation of the 5'UTR (grey box) and first exon (black box) of the *5PTase13* gene, showing the site of insertion and orientation of the T-DNA in the *5ptase13-3* mutant (SALK_083676). Arrows indicate the location of the F1 and R4 *5ptase13*-specific primers and the LBd1 primer annealing specifically at the left border of the T-DNA. **(c)** PCR-based genotyping of a *5ptase13-3* homozygous insertion line. The location of the F1, R4 and LBd primers is shown in (b). **(d)** RT-PCR analysis of *5PTase13* expression in Col-0 wild-type (WT3) and mutant (*5ptase13-3*) plants. The location of the F1 and R4 primers used is shown in (b). Expression of the *ACTIN* (*ACT*) gene was used as a loading control.

To examine the effect of the T-DNA insertion on the expression of the disrupted *5PTase13* gene, RT-PCR analysis of *5PTase13* expression in the *5ptase13-3* mutant line was performed. Using gene-specific primers flanking the insertion site (Figure 9b, Table 2), no expression was detected in the mutant (Figure 9d). It therefore appears that *5ptase13-3* is a true genetic null mutant, at least incapable of producing a full-length *5PTase13* transcript.

3. Phenotypical analysis of the *5PTase13-3* loss-of-function mutant

Bearing in mind future studies to investigate potential different functions of the *5PTase13* isoforms in the plant, it was imperative to check whether disruption of the *5PTase13* gene would lead to any evident phenotype. Thus, phenotypical analyses were conducted on the *5ptase13-3* loss-of-function

mutant alongside the previously reported *5ptase13-2* mutant (Ananieva et al., 2008). Since both these mutants harbor the T-DNA insertion in the first exon, we wanted to evaluate whether both would display the same phenotypes despite the latter being a leaky mutant. Phenotypical analyses included germination rates under normal and low light, in the presence or absence of glucose or ABA, as well as root growth under low light conditions.

3.1. Effect of glucose and ABA on seed germination under normal and low light conditions

The germination of *5ptase13-3* and *5ptase13-2* mutant seeds on 0.5 X MS control medium under normal or low light conditions occurred at the same time as their respective wild types (WT3 and WT2) with full germination being attained one day after stratification (Figure 10a and 11a). The presence of 5% glucose in the media led to a delay in germination for all genotypes, with full germination rates occurring only around day 4 after stratification for *5PTase13-3* and WT3, and around day 6 for *5PTase13-2* and WT2, both under normal and low light conditions (Figure 10b and 11b). Although no differences in germination were perceived between *5ptase13-3* and WT3, the presence of glucose produced a slight delay in the germination of *5ptase13-2* mutant seeds, with the mutant never reaching the same germination rate as its corresponding wild type (WT2). This very slight delay occurred under both normal and low light conditions.

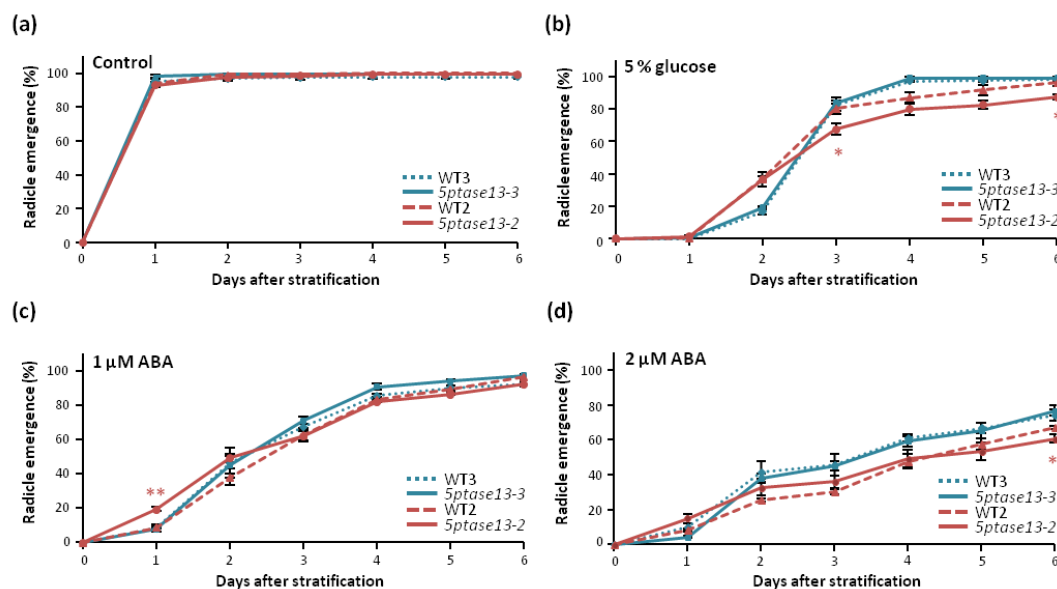


Figure 10. Germination of *5ptase13-3* and *5ptase13-2* mutant seeds under normal light

Germination (radicle emergence) rates of *5ptase13-3* (blue lines) and *5ptase13-2* (red lines) mutant seeds, as well as of the corresponding Col-0 wild-type (dashed lines) seeds, grown under standard light conditions (100 $\mu\text{mol s}^{-1} \text{m}^{-2}$) in (a) control conditions or in the presence of (b) 5% glucose, (c) 1 μM ABA and (d) 2 μM ABA (means \pm SE, $n=3$). Asterisks indicate significant differences between the *5ptase13-3* or *5ptase13-2* mutants and their corresponding wild-types (* $p < 0.05$; ** $p < 0.01$) according to Student's *t*-test.

The exogenous application of 1 μM and 2 μM of ABA delayed germination of wild-type and mutant seeds to a similar extent under both light intensity conditions (Figures 10c-d, 11c-d). The only exception was for the *5ptase13-2* mutant, which in the presence of 1 μM ABA under normal light in the first two days was less affected than the wild type (WT2) (Figure 10c). All genotypes reached their full germination rate six days after stratification in the presence of the lower concentration of ABA in normal light conditions (Figure 10c) however, under low light, a full germination rate was attained slightly earlier — *5ptase13-3* and WT3 at day 4 and *5ptase13-2* and WT2 at day 5 after stratification (Figure 11c). The delay in germination caused by ABA is more drastic in the presence of a higher concentration of this hormone (2 μM). As can be seen in Figures 10d and 11d, with the exception of *5ptase13-3* and WT3 under low light that were able to be fully germinated by day 6, under both light conditions neither of the genotypes was ever able to reach a 100% germination rate in this same 6-day time frame.

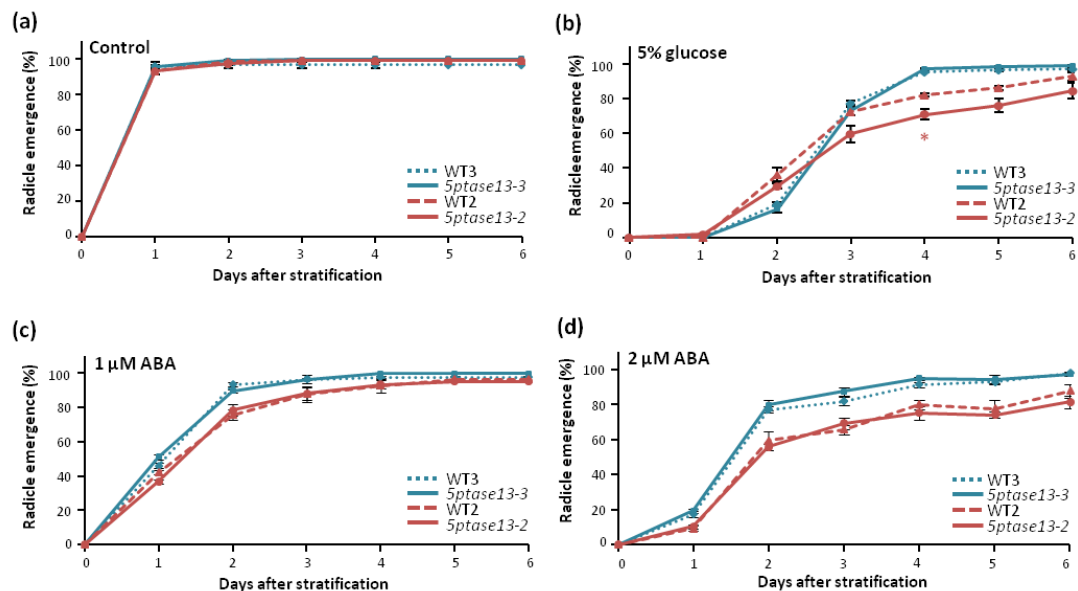


Figure 11. Germination of *5ptase13-3* and *5ptase13-2* mutant seeds under low light

Germination (radicle emergence) rates of *5ptase13-3* (blue lines) and *5ptase13-2* (red lines) mutant seeds, as well as of the corresponding Col-0 wild-type (dashed lines) seeds, grown under low light (40 $\mu\text{mol s}^{-1} \text{m}^{-2}$) in (a) control conditions or in the presence of (b) 5% glucose, (c) 1 μM ABA and (d) 2 μM ABA (means \pm SE, $n=3$). Asterisks indicate significant differences between the *5ptase13-3* or *5ptase13-2* mutants and their corresponding wild-types (* $p < 0.05$) according to Student's *t*-test.

These results indicate that the *5ptase13-3* and *5ptase13-2* mutants are neither affected by glucose nor ABA, either under normal or low light conditions.

3.2. Root growth under low light conditions

Root growth of the two *5PTase13* mutants (*5ptase13-3* and *5ptase13-2*) and their respective wild types (WT3 and WT2) was examined under low light conditions. Importantly, both mutants exhibited a significantly reduced root growth, both at 4 and 6 days after seed stratification (Figure 12), suggesting that under these conditions the 5PTase13 protein promotes root growth.

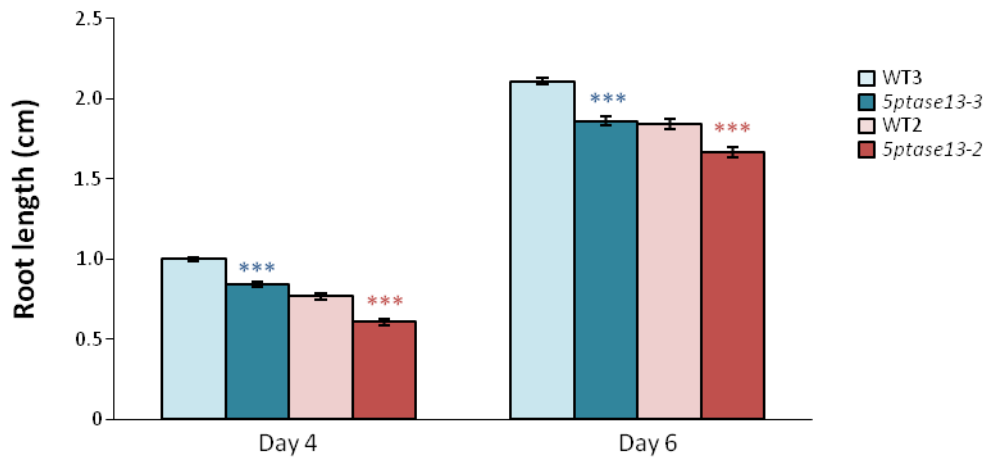


Figure 12. Root growth of the *5ptase13-3* and *5ptase13-2* mutants under low light

Root length of *5ptase13-3* and *5ptase13-2* mutant seedlings, as well as of the corresponding Col-0 wild-type seedlings, grown under low light conditions ($40 \mu\text{mol s}^{-1} \text{m}^{-2}$) and scored 4 or 6 days after stratification (means \pm SE, $n=76-102$). Asterisks indicate significant differences between the *5ptase13-3* or *5ptase13-2* mutants and their corresponding wild-types (*** $P<0.001$) according to Student's *t*-test.

4. Functional relevance of each 5PTase13 isoform in the *sr45-1* background

It has been previously reported that the *SR45* loss of function mutant, *sr45-1*, exhibits hypersensitivity to glucose during early seedling development (Carvalho et al., 2010). Moreover, the splicing pattern of the *5PTase13* gene is markedly altered in the *sr45-1* loss of function mutant, where the abundance of the longer splice variant (*5PTase13.1*) is considerably higher (Figure 13 and Carvalho et al., submitted). This led us to hypothesize a potential role of the 5PTase13 phosphatase in SR45 control of glucose responses. To test this hypothesis, we investigated the potential *in vivo* function of the two 5PTase13 protein isoforms in the *sr45-1* mutant background. To this end, we generated transgenic plants overexpressing either GFP-tagged *5PTase13.1* or *5PTase13.2* under the control of the 35S promoter in the *sr45-1* background (*sr45-1/35S:5PTase13*) (Figure 13) in order to assess their germination and early seedling development under glucose conditions (Figure 14).

The expression levels of the splice variant in transgenic and wild-type plants were compared by RT-PCR. The amplified bands were then quantified and the ratio between the *5PTase13.1* and

5PTase13.2 isoforms calculated (Figure 13). As expected, the four independent transgenic lines overexpressing *5PTase13.1* (lines #1, #5, #8 and #9) exhibited a higher expression of this isoform, reaching up to four times more expression than in the wild type (Figure 13). On the other hand, expression of the second isoform (*5PTase13.2*) in the lines overexpressing this isoform (lines #4, #7, #9 and #13) was higher when compared to the wild type. With the exception of the *sr45-1/35S:5PTase13.2* line #4, which shows a more similar expression ratio with the wild type, all the other three exhibited up to four times higher expression of *5PTase13.2* transcript than the wild-type (Figure 13).

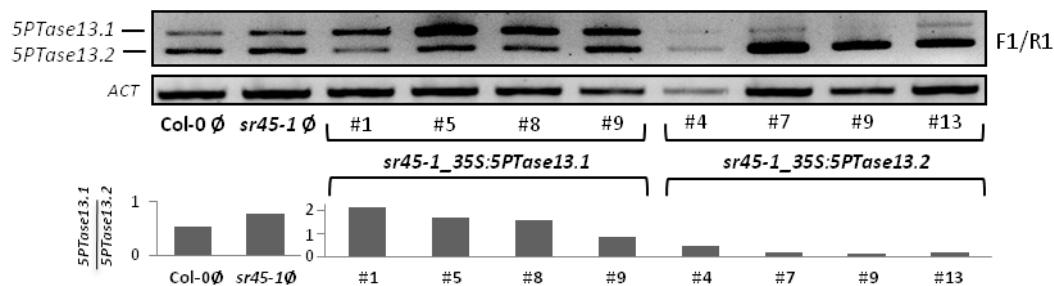


Figure 13. *5PTase13* transcript levels in *sr45-1* mutant plants expressing the individual *5PTase13* splice variants

RT-PCR analysis of the transcript levels of the two *5PTase13* splice variants (*5PTase13.1* and *5PTase13.2*) in plantlets of wild-type (Col-0) and *SR45* mutant (*sr45-1*) plants transformed with the GFP empty vector, as well as of 8 independent lines expressing either the *5PTase13.1* (*35S:5PTase13.1*, lines #1, #5, #8 and #9)- or the *5PTase13.2* (*35S:5PTase13.2*, lines #4, #7, #9 and #13)-GFP-tagged wild-type coding sequence under the control of the 35S promoter in the *sr45-1* mutant background. The location of the F1 and R1 primers used is shown in Figure 1. Expression of the *ACTIN* (*ACT*) gene was used as a loading control. Bands were quantified and the histogram shows the *5PTase13.1/5PTase13.2* ratios in each transgenic line.

Under normal growth conditions, seed germination and early seedling development (cotyledon greening and expansion) of wild-type (Col-0) and mutant (*sr45-1*) plants transformed with the GFP empty vector, and overexpression lines, *sr45-1/35S:5PTase13.1* (lines #1, #5, #8 and #9)- and *sr45-1/35S:5PTase13.2* (lines #4, #7, #9 and #13)-GFP are similar to each other (Figure 14). When the seeds were germinated in medium supplemented with 3% glucose, no significant differences were observed in radical emergence (germination) between the wild type, *sr45-1* or transgenic lines. However, it is clear that the *sr45-1* and all transgenic lines display a delay in early seedling development when compared with the Col-0 wild type (Figure 14). When each of the *5PTase13* isoforms is overexpressed in the *sr45-1* mutant background, the glucose hypersensitivity is considerably enhanced — all transgenic lines show a higher delay in cotyledon greening when compared with the *sr45-1* mutant, especially *sr45-1/35S:5PTase13.1* line #5, and *sr45-1/35S:5PTase13.2* lines #4 and #9 (Figure 14). Regarding cotyledon expansion, only transgenic

lines #5 and #9, overexpressing *5PTase13.1*, and transgenic line #9, overexpressing *5PTase13.2*, present a significant delay when compared with the *sr45-1* mutant (Figure 14).

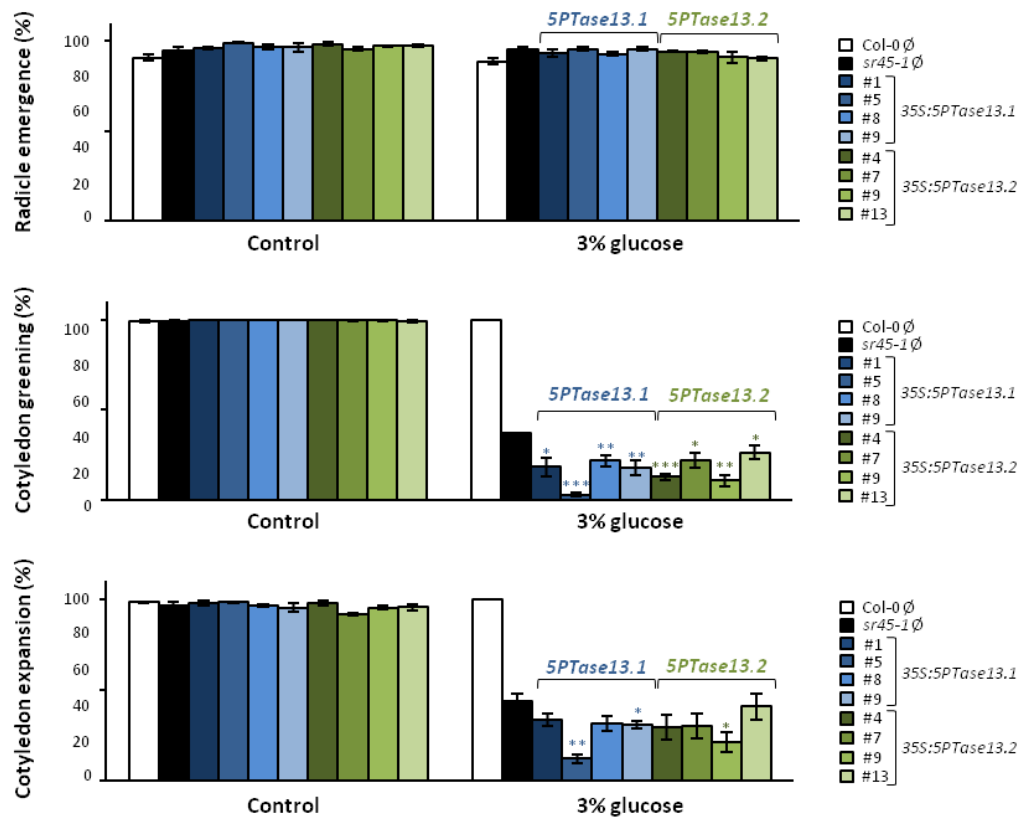


Figure 14. Glucose-related phenotypes of transgenic plants expressing the individual *5PTase13* splice variants in the *sr45-1* mutant background

Germination, cotyledon greening and cotyledon expansion rates, scored 7 days after stratification, of Col-0 \emptyset (white bars), *sr45-1* \emptyset (black bars), 35S:*5PTase13.1* (lines #1, #5, #8 and #9, blue bars) and 35S:*5PTase13.2* (lines #4, #7, #9 and #13, green bars) seedlings grown under control conditions or in the presence of 3% glucose (means \pm SE, $n=3$). Asterisks indicate significant differences between the 35S:*5PTase13.1* or 35S:*5PTase13.2* transgenic lines and the *sr45-1* mutant transformed with the empty vector (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) according to Student's *t*-test.

These results show that 35S promoter-driven overexpression of *5PTase13.1* and *5PTase13.2* in the *sr45-1* background results in a stronger phenotype than the *sr45-1* mutation alone regarding glucose hypersensitivity during early seedling development, with the effect being less evident regarding cotyledon expansion. These results also indicate that both *sr45-1:5PTase13.1* and *sr45-1:5PTase13.2* respond similarly to glucose.

5. Subcellular localization of GFP-tagged SR45 and 5PTase13 in Arabidopsis roots

Confocal microscopy analysis of transgenic Arabidopsis roots stably expressing GFP alone or the SR45.1-GFP fusion in the Col-0 background under the control of 35S promoter is shown in Figure 15. As expected, GFP alone was detected in the cytoplasm (Figure 15a) while the GFP-SR45.1 fusion protein was found exclusively in the nucleus (Figures 10b-d), indicating targeting to the nuclear compartment. The SR45.1 protein is either distributed diffusely in the nucleoplasm (Figures 15b-c) or grouped in bright round concentrated areas, called speckles, as shown in a close-up of the nuclei (Figure 15d).

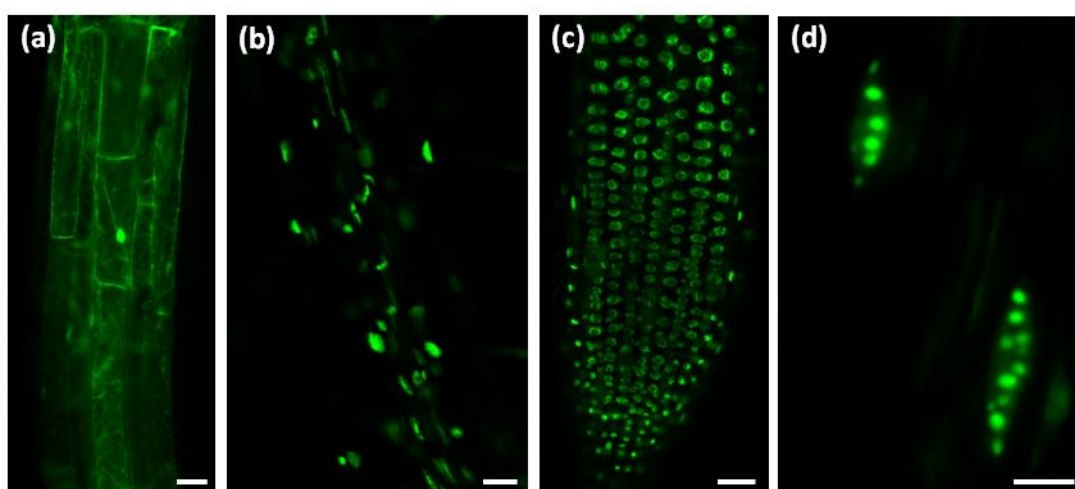


Figure 15. Subcellular localization of the SR45.1 isoform in Arabidopsis roots

Representative confocal laser scanning microscopy images of root epidermal cells of transgenic Arabidopsis 7-d old seedlings stably expressing either GFP alone (a) or the SR45.1-GFP fusion protein (b-d) under the control of the 35S promoter in the wild-type (Col-0) background. (a-b) primary root; (c) primary root tip; (d) nuclei close-up. The GFP signal is visualized by green coloration. Scale bars, 25 μ m (a-c) and 10 μ m (d).

To investigate the subcellular localization of the 5PTase13.1 and 5PTase13.2 isoforms, we also performed confocal microscopy analysis on transgenic *sr45-1/35S:5PTase13* roots. In contrast to the fluorescence signal detected with GFP alone, which again as expected localized to the cytoplasm (Figures 16a-b), the fluorescence signal emanating from the two fusions was significantly lower. From our observations, it appears that both isoforms also localize to the cytoplasm. However, in the case of the 5PTase13.1 isoform, the signal seems to be mostly associated with the plasma membrane rather than being uniformly distributed inside the root cells. As illustrated in Figures 16g-i, and in accordance with software predictions, we cannot exclude the possibility that both isoforms also localize to the nucleus.

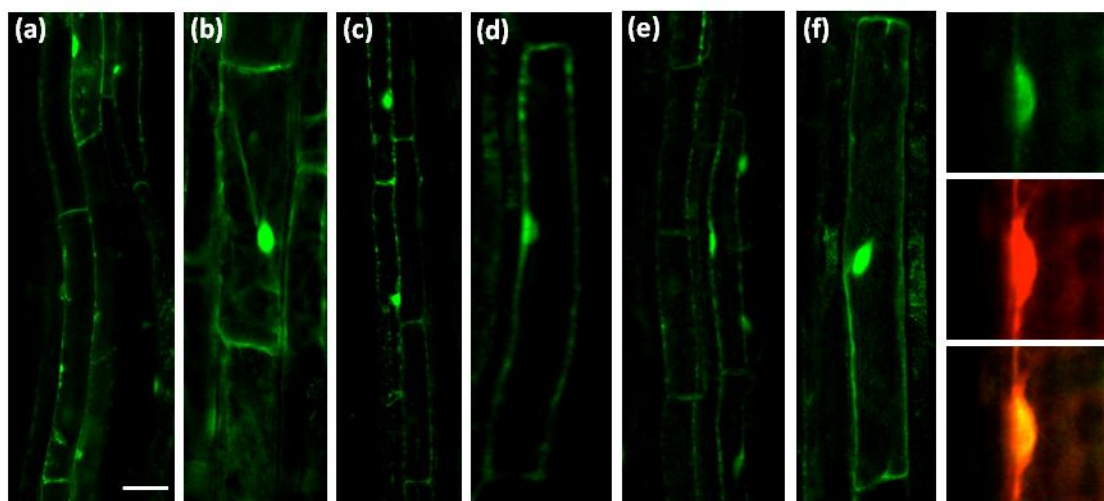


Figure 16. Subcellular localization of the 5PTase13.1 and 5PTase13.2 isoforms in Arabidopsis *sr45-1* mutant roots

(a-f) Confocal laser scanning microscopy images of epidermal cells of transgenic Arabidopsis roots stably expressing either GFP alone **(a, b)** or the 5PTase13.1-GFP **(c, d)** or 5PTase13.2-GFP **(e, f)** fusion proteins under the control of the 35S promoter in the *sr45-1* mutant background. The GFP signal is visualized by green coloration. Scale bars, 25 μ m.

(g-i) Confocal laser scanning microscopy images of an epidermal cell of transgenic Arabidopsis roots stably expressing the 5PTase13.2-GFP fusion **(g)** under the control of the 35S promoter in the *sr45-1* mutant background. The cell wall is stained by propidium iodide **(h)** and the merged image is shown **(i)**. The GFP and propidium iodide signals are visualized by green and red coloration, respectively. Scale bars, 10 μ m.

In order to better understand the subcellular localization of 5PTase13, we transiently expressed each one of the isoforms in Arabidopsis wild-type protoplasts (Figure 17). We used GFP alone as a cytoplasmic control (Figure 17a-c) and SR45.1-GFP as a nuclear control (Figure 17d-f). From the images obtained we can observe that both 5PTase13 isoforms seem to be localized to the cytoplasm. However, whereas for 5PTase13.2 there is a clear visualization of the cytoplasmic strands, once again confirming its cytoplasmic localization (Figure 17k), 5PTase13.1 presents a reticulate network pattern of GFP fluorescence (Figure 17h), which is very typical of endoplasmatic reticulum localization. Future co-localization studies will help confirm these preliminary results.

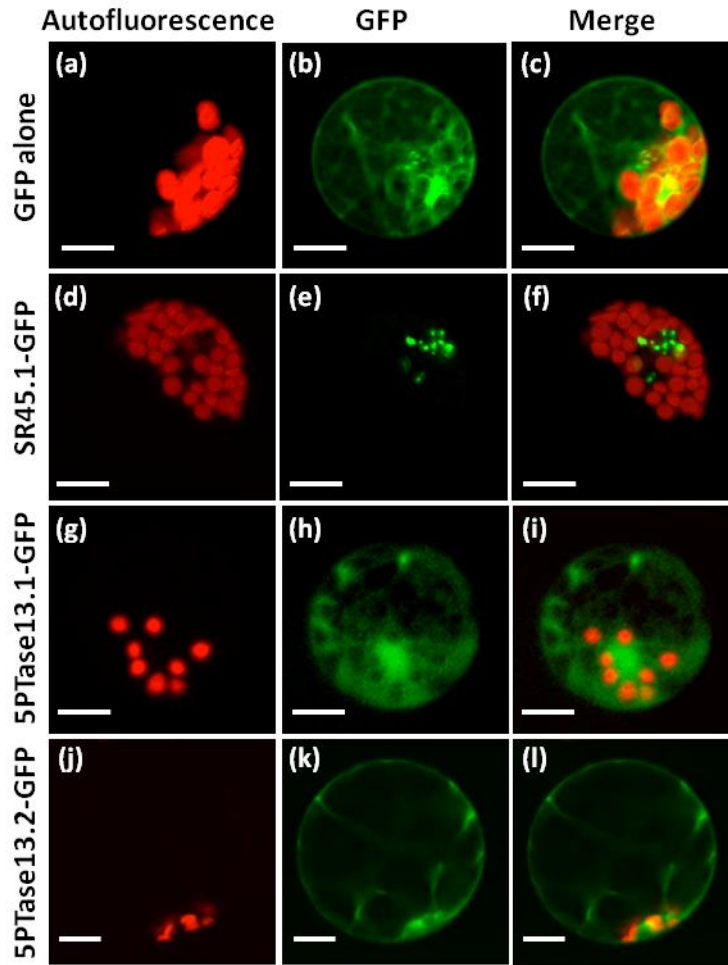


Figure 17. Subcellular localization of SR45.1 as well as 5PTase13.1 and 5PTase13.2 isoforms in protoplasts

(a-i) Confocal laser scanning microscopy images of mesophyll protoplasts of *Arabidopsis* transiently expressing either GFP alone (a,b,c), the SR45.1-GFP (d,e,f) or the 5PTase13.1-GFP (g,h,i) or 5PTase13.2-GFP (j,k,l) fusion proteins under the control of the 35S promoter. Chloroplasts were visualized by red chlorophyll autofluorescence (a,d,g,j). The GFP signal is visualized by green coloration (b,e,h,k). Merge image is shown (c,f,i,l). Scale bars, 10 μ m.

IV. DISCUSSION

The existing reports on the functional characterization of the 5PTase13 phosphatase have shown this protein to play important roles in plant development as well as in hormone and light signaling pathways.

The *5PTase13* gene undergoes alternative splicing, generating two distinct splice variants as a result of the retention of intron 6 in its pre-mRNA. The resulting protein isoforms differ in only 34 amino acids inserted into the 5PTase13.1 splice form. The fact that the 34 amino-acid stretch is included in the catalytic domain of the phosphatase could potentially affect the conformation and properties of the catalytic site of the enzyme. On the other hand, although the alternative segment contains no specific recognizable protein motif, it includes an extra N-myristoylation site, which could conceivably affect the activity and/or stability of the protein. In fact, preliminary data in the lab shows that the 5PTase13.1 isoform is less expressed than 5PTase13.2. One hypothesis is that the intron retention event would give rise to a less stable protein isoform.

Expression pattern analysis by semi-quantitative RT-PCR showed that both *5PTase13* splice variants are expressed in Arabidopsis seedlings and plantlets of different ages, albeit at lower levels in young seedlings, as well as ubiquitously in different aerial organs and roots. This is in agreement with previous reports where total *5PTase13* expression was detected in young seedlings, inflorescence apices, roots, stems and mature leaves (Lin et al., 2005; Zhong & Ye, 2004). Furthermore, we observed that the constitutively-spliced *5PTase13.2* is the most abundant transcript in all the analyzed seedlings, plantlets and organs. The ratio between the two splice variants is fairly constant, the only exception being for 14 day-old plantlets, where the expression of the *5PTase13.1* transcript is markedly higher compared to seedlings and other different-aged plantlets or different organs.

Interestingly, the Arabidopsis *5PTase13* gene has been previously reported to be significantly induced by ABA and wounding and slightly induced by cold and salt treatment, indicating that this phosphatase might play a role in stress responses (Zhong & Ye, 2004). Indeed, the functional characterization of two *5PTase13* loss-of-function mutants, *5ptase13-1* and *5ptase13-2*, has shown them to display altered responses to ABA and sugar stress (Ananieva et al., 2008; Lin et al., 2005). Here, we were interested in investigating whether the production of the two *5PTase13* splice variants had an *in vivo* functional impact in plant sugar and ABA responses. We first obtained the previously characterized *5ptase13-1* and *5ptase13-2* mutants, but RNA analyses showed them to still express significant levels of the *5PTase13* mRNAs. For this reason, we isolated a new T-DNA mutant, *5PTase13-3*, which appears to represent a true genetic null mutant, at least incapable of encoding a full-length 5PTase13 protein. Nevertheless, we were unable to reproduce most of the previously

reported ABA and glucose phenotypes (Ananieva et al., 2008), neither in the new isolated knockout mutant, *5ptase13-3*, nor in the *5ptase13-2* allele. During their development, seeds undergo changes in both ABA content and sensitivity, which are well known to affect sugar responses. ABA levels reach their highest during mid- and late-seed maturation stages, which is what determines seed dormancy. Hence, freshly harvested seeds usually show high levels of dormancy that are gradually released during dry storage (after-ripening). The duration of seed ripening and the temperature or period of time during which the seeds are stored are very important in their germination ability, and the fact that they might not be exactly the same as in Ananieva et al. (2008) and Lin et al. (2005), could be one explanation for the lack of evident germination phenotypes in the *5PTase13* mutants we characterized. In fact, it could be possible that the reported ABA and glucose *5PTase13* insensitive phenotypes are only observed in freshly-harvested seeds. For instance, Dekkers et al. (2008) showed that *abi3* mutants display a glucose insensitive phenotype, when in earlier studies (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000) no clear glucose insensitivity had been observed for this mutant. This difference could be due to the seed material, as it was observed that seed sensitivity to sugars was different between freshly-harvested seeds and after-ripened seeds (Dekkers, et al., 2008). Germination phenotypes can thus sometimes be masked by differences in the seed after-ripening period.

On the other hand, *5ptase13-3* mutants grown under low light exhibit shorter roots, which is in accordance with what was previously reported for the *5ptase13-1* and *5ptase13-2* mutants by Ananieva et al. (2008). Interestingly, these same authors verified that when exogenous sugar was added to the growth medium, there were no differences in the root length of the *5PTase13* mutants compared to the wild type. Light, through photosynthesis, provides the energy required for plant growth. When light intensity is lower, less energy is available to the plant. These sessile organisms have therefore evolved adaptive mechanisms that allow them to adjust their growth and developmental processes in response to changes in light intensity. Our results suggest that the 5PTase13 phosphatase plays a role in promoting root growth under low light and, consequently, low energy conditions. Ananieva et al. (2008) showed that loss of 5PTase13 function affects SnRK1 activity, and that this impact differs depending on nutrient availability – in *5PTase13* mutant seedlings grown in the same conditions as ours, SnRK1.1 activity is decreased when compared with wild-type seedlings. These results are in accordance with the observation that under low energy conditions, SnRK1.1 silencing lines exhibit reduced root growth (Baena-González et al., 2007). Another explanation for this root phenotype could be related to Ins(1,4,5)P₃ signaling. Salinas-Mondragon et al. (2010) showed that transgenic plants constitutively expressing the human Type I inositol polyphosphate-5-phosphatase (InsP 5-PTase) exhibit increased root length when grown

under low light conditions. This human Type I phosphatase specifically hydrolyzes soluble inositol phosphates (e.g. $\text{Ins}(1,4,5)\text{P}_3$), therefore its heterologous expression in *Arabidopsis* reduced $\text{Ins}(1,4,5)\text{P}_3$ in roots to less than 5% of wild-type levels (Salinas-Mondragon et al., 2010). As the *Arabidopsis* 5PTase13 hydrolyzes $\text{Ins}(1,4,5)\text{P}_3$, we would expect the *5ptase13* mutants to display higher levels of this secondary messenger. This could explain the decreased root growth observed in the *5ptase13* mutants, which is in agreement with the above mentioned increased root phenotype observed by Salinas-Mondragon et al. (2010) for seedlings with lower levels of $\text{Ins}(1,4,5)\text{P}_3$. Although Ananieva et al. (2008) show that the *5ptase13-1* and *5PTase13-2* mutants do not differ significantly from wild-type seedlings in their $\text{Ins}(1,4,5)\text{P}_3$ levels, their results were obtained with dark-grown seedlings. Therefore, it would be interesting to check $\text{Ins}(1,4,5)\text{P}_3$ levels in seedlings grown under low light conditions where the root phenotype is observed. Finally, future work should elucidate whether either or both isoforms are able to rescue root growth under low light conditions, thus unequivocally assessing their functional significance in root growth.

Importantly, we show here that the *5PTase13* pre-mRNA gene is an endogenous target of the SR-like SR45 splicing factor, confirming previous results in the lab by Carvalho et al. (submitted). The SR45 protein has been previously reported to negatively regulate sugar and ABA signaling (Carvalho et al., 2010) and subsequent epistatic analyses showed that the function of the SR45 splicing factor in glucose signaling largely depends on the energy sensing SnRK1.1 protein kinase, which is consistent with the *sr45-1* mutant containing higher levels of SnRK1.1, particularly under glucose conditions (Carvalho et al., submitted). Interestingly, Ananieva et al. (2008) have described the 5PTase13 phosphatase to be involved in nutrient/energy signaling, interacting with and stabilizing the SnRK1.1 energy sensor. The fact that the splicing pattern of the *5PTase13* gene is altered in the *sr45-1* mutant provides a mechanistic link between SR45 function and the modulation of SnRK1.1 levels in response to sugars. Therefore, we were interested in investigating whether any or both of the 5PTase13 isoforms would affect the *sr45-1* glucose phenotypes. For this purpose, we generated *sr45-1/35S:5PTase13* transgenic plants overexpressing each of the isoforms in the *sr45-1* mutant background, and evaluated their germination and early seedling development under glucose conditions. We observed that overexpression of either *5PTase13.1* and *5PTase13.2* in the *sr45-1* background results in enhanced glucose hypersensitivity during early seedling development when compared with the *sr45-1* mutant. Both *sr45-1/35S:5PTase13.1* and *sr45-1/35S:5PTase13.2* respond similarly to glucose, indicating that the two isoforms do not influence differentially the glucose hypersensitive phenotype. However, to conclusively assess a link between 5PTase13 and SR45 function, it will be important in the future to analyze the effects of each 5PTase13 splice form in the wild-type background.

We could not find any correlation between the expression levels of the *5PTase13* splice variants and the intensity of the phenotype under glucose conditions. For instance, the transgenic *sr45-1/35S:5PTase13.1* line #5 showed the highest delay in cotyledon greening, but displayed a very similar *5PTase13.1/5PTase13.2* ratio to that observed for the *sr45-1/35S:5PTase13.1* line #8. Nevertheless, the latter showed a much weaker phenotype than the first. In addition, from the four investigated transgenic lines for isoform 1, *sr45-1/35S:5PTase13.1* line #1, which expresses the highest levels of the *5PTase13.1* transcript, was the one exhibiting less differences when compared to the *sr45-1* mutant. On the other hand, from all the transgenic lines overexpressing isoform 2, the *sr45-1/35S:5PTase13.2* line #4, showing the most similar *5PTase13.1/5PTase13.2* ratio to the wild type, is the one that displayed the stronger phenotype. To obtain a more clear and reliable expression quantification of *5PTase13* transcript in each line, RT-qPCR analyses should be undertaken. In addition, it will also be important to check the protein levels of the GFP-tagged *5PTase13* transgenic lines, as these do not always correlate with transcript levels.

Finally, we investigated whether the two *5PTase13* isoforms could exhibit different subcellular localization. Again, we used transgenic *sr45-1/35S:5PTase13* roots, overexpressing either the *5PTase13.1* or the *5PTase13.2* splice forms. Confocal microscopy analysis showed that both isoforms appear to be located in the cytoplasm, although the GFP signal from the *5PTase13.1* isoform seemed mostly associated with the plasma membrane rather than being uniformly distributed inside the root cells. In contrast to our results, Ananieva et al. (2008) have reported *5PTase13.2* to be located in the nucleus of *Arabidopsis* seedlings, and software predictions also support the nuclear localization for both isoforms. In root cells it is sometimes very difficult to distinguish between cytoplasmic and nuclear localization due to the large size of the central vacuole. In addition, due to its small size, GFP can also enter the nucleus (reviewed in Hanson & Kohler, 2001). Therefore our results cannot either confirm or rule out the possibility that both isoforms localize to the nucleus as well. In order to further investigate the subcellular localization of the *5PTase13* isoforms, we transiently expressed *5PTase13.1* and *5PTase13.2* in protoplasts. These results confirm a cytoplasmic localization for both isoforms however, *5PTase13.1* presented a fluorescence pattern that is similar to that of endoplasmatic reticulum localization. Again, we could not conclude about a nuclear localization and further studies need to be done.

Together, the present work has provided a first null mutant allele for the *5PTase13* gene that displays the previously reported root growth phenotypes. Subcellular localization studies suggest that both *5PTase13* splice forms are localized to the cytoplasm. Finally, our phenotypical analysis of transgenic *sr45-1/35S:5PTase13* plants suggest that alternative splicing of the *5PTase13* gene does not play a role in *SR45* control of glucose responses.

V. CONCLUSIONS AND FUTURE PERSPECTIVES

Results stemming from this work indicate that the *5PTase13* gene is ubiquitously expressed in Arabidopsis and that, although not involved in seed germination, the encoded inositol polyphosphate 5-phosphatase 13 is required for root elongation under limiting light conditions. Our results also show that both *5PTase13* splice forms enhance the glucose oversensitivity conferred by the *sr45-1* mutation. However, as the two isoforms have a similar effect, alternative splicing of 5PTase13 does not appear to play a role in SR45 control of sugar responses. Finally, regarding the subcellular localization of each of the 5PTase13 isoforms, our results suggest that both isoforms are localized to the cytoplasm. From our root assays it seemed that the 5PTase13.1 isoform could be more associated with the plasma membrane, but in protoplasts it has a pattern similar to endoplasmic reticulum localization. Although not conclusively supported by our data, nuclear localization cannot be ruled out.

Future studies will focus on the in vivo functional analysis of the 5PTase13 splice forms. One of the ways to address this will be by means of their individual overexpression, under the control of the 35S promoter or the 5PTase13 endogenous promoter, in the *5ptase13-3* background followed by assessment of complementation of the mutant's root phenotype. On the other hand, independent overexpression of each isoform in the *sr45-1 5PTase13-3* double mutant background could be more elucidative of their participation in the *sr45-1* glucose phenotype. Although our transgenic plants only overexpress one of the 5PTase13 isoforms, the fact that the other is still present at wild-type levels could complicate interpretation of the results. Finally, independent transient expression of the two 5PTase13 isoforms in tobacco leaves or colocalization with various different markers (i.e. nuclear, endoplasmic reticulum, plasma membrane) in Arabidopsis protoplasts should allow for a more conclusive analysis of the subcellular localization of each of the 5PTase13 splice forms.

VI. REFERENCES

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